The Quantitation of Antibodies to Idiotypic Determinants of Anti-HLA Antibodies in Renal Transplant Patients

Thesis submitted by

Tsang Kam Sze, Kent

in partial fulfilment for the Degree of

Master of Philosophy

in

Division of Clinical and Pathological Sciences

The Chinese University of Hong Kong

June, 1992

Abstract of Thesis

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Abstract

Blood transfusion is known to improve graft survival in renal transplant patients. The exact mechanism(s) by which blood transfusion confers this improved graft survival is still unknown, but some postulate that the presence of antibodies to idiotypic determinants of anti-HLA antibodies plays an important role. The aim of this project is to study the relationship between transfusion and the level of anti-idiotypic antibodies (anti-Ids) against anti-HLA antibodies in renal transplant patients and its effect on graft survival.

An enzyme immunoassay based upon the ELISA principle was developed to detect anti-Ids to 9 anti-HLA antibodies in 101 renal transplant patients (48 cadaveric donors [CD] and 53 living donors [LD]). Anti-HLA antibodies were purified by affinity chromatography and pepsinized to produce $F(ab')_2$. Purified $F(ab')_2$ were then used to coat microplate, and the binding of the corresponding anti-Ids in serum samples to the anti-HLA $F(ab')_2$ was detected spectrophotometrically. The optimal condition of the assay was empirically obtained.

The highest anti-Ids level found in each patient was measured. Renal transplant patients were categorized into various groups, namely: LD and CD, transfused and non-transfused, and the anti-Ids level of each group were compared with normal controls and also with each other.

In 26 transplant patients whose blood samples were available both before and after transplant, the temporal study showed that most patients reached their peak anti-Ids level within the first 100 days after transplantation.

In comparison with normal controls, higher level of anti-Ids ($p < 0.05$) was found in transplant patients. LD transplant patients were less likely to have significantly high anti-Ids than CD transplant patients. Transplant patients with pre- and post-transplant transfusion had anti-Ids notably increased ($p < 0.05$), but the peroperative transfused group had comparable level with the normal controls ($p > 0.1$).

High but not significantly elevated anti-Ids level was found in transfused transplant patients than non-transfused transplant patients ($p > 0.1$). There was also no significant increase of anti-Ids in transplant patients with various transfusion modalities ($p > 0.1$).

Graft rejection occurred more frequently in non-transfused than transfused CD kidney recipients ($p < 0.05$), but there was no significant difference encountered in LD kidney recipients ($p > 0.05$). The average rejection episodes per patient were notably higher in non-transfused transplant patients ($p < 0.01$).

The graft failure rates were 5.66% (3/52) and 20.83% (10/48) for LD and

CD kidney recipients respectively. The overall failure rate was 12.87% (13/101). CD kidney recipients were 3.68 times more vulnerable to graft failure than their LD transplant counterparts (10/48 in CD transplant versus 3/53 in LD transplant). Chi-squared analysis showed that graft survival was related to transfusion ($p < 0.05$).

This study demonstrated the level of anti-Ids was higher in transplant patients than normal subjects, and an association between blood transfusion and a higher level of anti-Ids. It also showed that transfused transplant patients had lesser rejection episodes and better renal graft survival.

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List of Abbreviations

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Ab_1	=	idiotype
Ab_2	=	anti-idiotype
AHG	=	goat anti-human IgG
Anti-Ids	=	anti-idiotypic antibodies
AP	=	alkaline phosphatase
BSA	=	bovine serum albumin
CD	=	cadaveric donor
CV	=	coefficient of variation
ELISA	=	enzyme linked immunosorbent assay
Fab	=	fragment of antigen binding
$F(ab')_2$	=	bivalent fragment of antigen binding
Fc	=	fragment of crystallisation
HLA	=	human leucocyte antigen
HRP	=	horseradish peroxidase
IgG	=	immunoglobulin G
LD	=	living donor
MLR	=	mixed lymphocyte reaction
OD	=	absorbance
OPD	=	o-phenylenediamine dihydrochloride
p	=	probability
PBS	=	phosphate buffer saline
PBST	=	phosphate buffer saline with Tween-20
PBSC	=	phosphate buffer saline with casein
pFc'	=	partial fragment of crystallisation
SD	=	standard deviation
SEM	=	standard error of mean
TcR	=	T-cell antigen receptor

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Chapter 1

Introduction

1. INTRODUCTION

1.1. Idiotype Network

The discovery of antigenic determinants associated structurally with the hypervariable region of the immunoglobulin molecule in human by Kunkel and co-workers[1] as well as similar findings in rabbits by Oudin and Michael[2] in 1963 led to the description of idiotype.

From Greek, "idios" means "of one's own or individual". An idiotype is a composite of idiotopes (antigenic determinants) located either in the complementarity determining regions of antibody combining sites (Figure 1.1) or the variable regions of antigen receptors of B and T lymphocytes. Whereas an antibody or a cellular receptor is regarded an immunogen, it possesses numerous antigenic determinants towards which specific antibodies can be elicited. Despite being idiosyncratic in nature, an idiotype may be shared, as a whole or in part, with other antibodies or cellular receptor molecules raised in response to a specifically immunological challenge within an individual or group of individuals, and leads to the semantic terminology of private (unique) and public (cross-reactive) idiotype respectively[3]. Antibodies with different antigenic specificity may share idiotypic determinants. On the contrary, antibodies to the same or slightly different epitodes on the same antigen may not necessarily share idiotype.

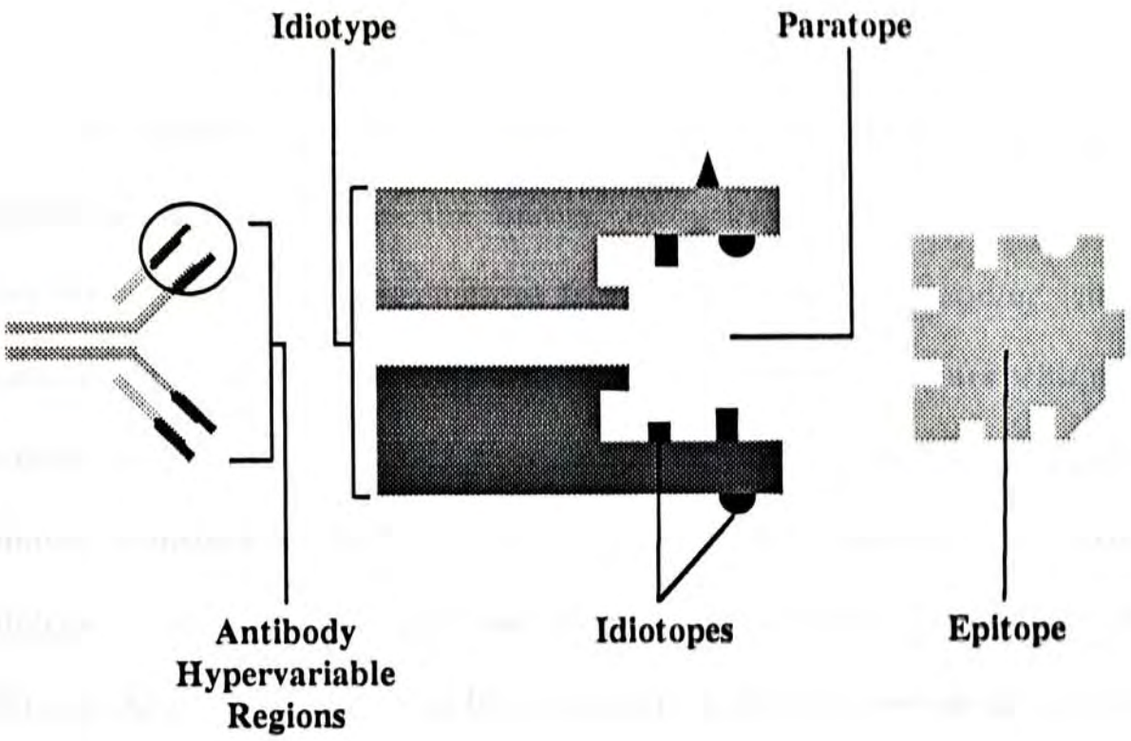


Figure 1.1. Schematic presentation of antibody nomenclature.

Idiotypes are recognized and defined by antibodies, B-cell antigen receptors (membrane-bound immunoglobulins) and T-cell antigen receptors (TcRs). These antibodies, B or T cells which direct against and bind to idiotypes are designated as anti-idiotypes. The distinction between idiotype and anti-idiotype is merely operational. Any idiotype of an antibody, or of an antigen receptor of B and T cells, can be employed as an immunogen to elicit a complementary humoral or cell-mediated immune response.

A regulatory idiotype network of the immune system in a state of suppression is based upon the mutual recognition between idiotype and complementary anti-idiotype, both as free antibody molecules and/or cellular receptors on B or T cells. Normally these complementary clones within the immune system are exceedingly large and simultaneously present to maintain immune homeostasis[4]; however, the equilibrium can be upset by foreign antigens. Extraneous antigens disrupt this balance through idiotypic-anti-idiotypic (Ab_1 - Ab_2) interactions by creating, from the immunology perspective, an excess of the internal image of antigens (Ab_2). Not only does the excess induce Ab_1 production against the externally derived antigens, but also the immoderate Ab_1 stimulates a second excessive internal image of the antigen. This second set of antibody, i.e. Ab_2, is then elicited against the idiotopes of Ab_1. This latter clone will in turn be regulated in the same fashion, creating what Jerne has called a web of V-domains (Figure 1.2), though, under normal condition, high concentration of an antibody directed against a particular

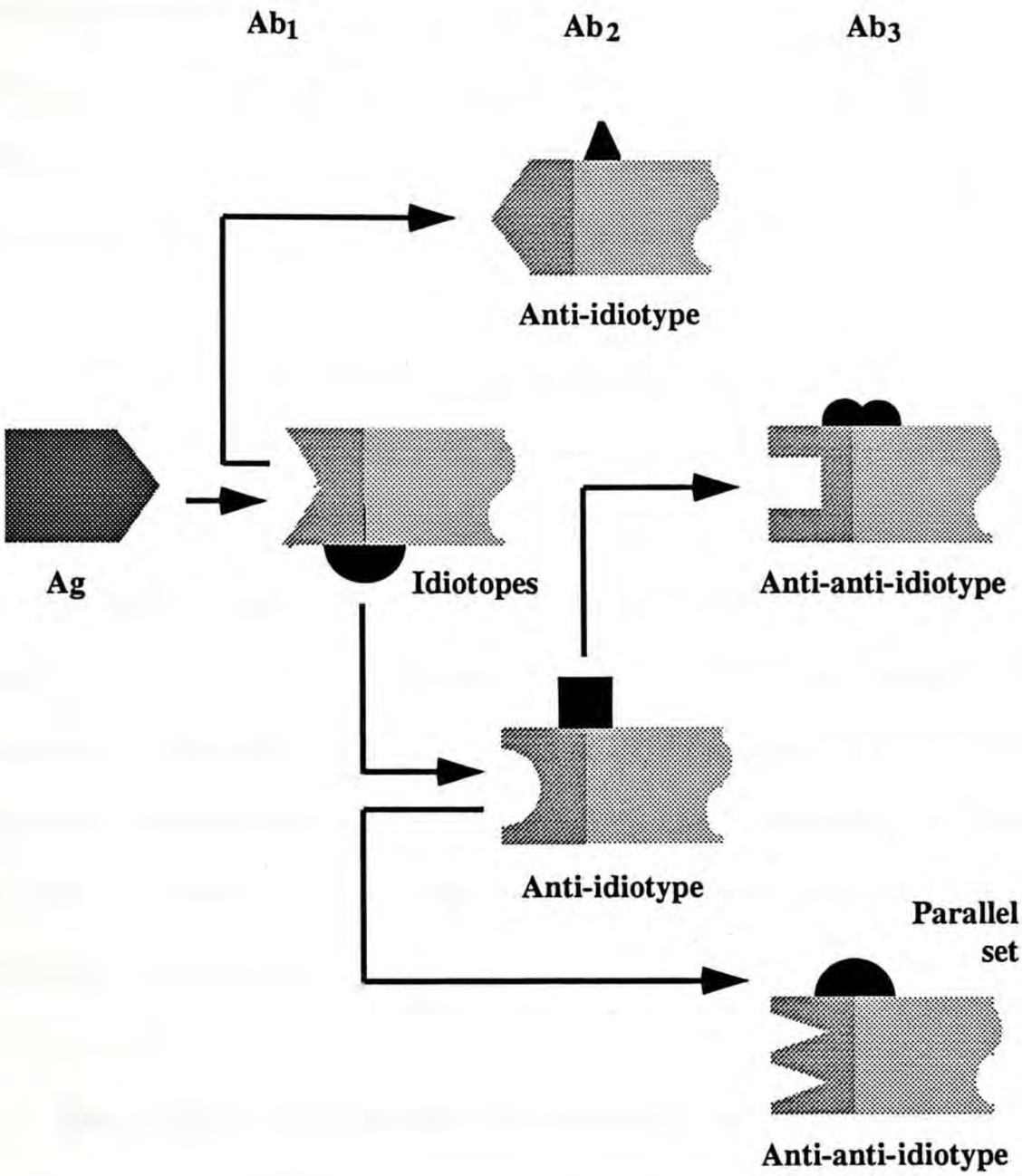


Figure 1.2. Jerne's network theory of the immune system.

unique idiotype is normally not present due to the extreme heterogenicity of the variable region of immunoglobulin to induce a particular anti-Id. This process continues through successive but decreasing stages of Ab_3, Ab_4 to Ab_n, till the system again retains a state of stable equilibrium. In such a way, the immune system is not only connected but accommodated within its repertoire of idiotypes and paratopes, a set of anti-idiotypes of all antigenic determinants that the system is likely to encounter in the external environment.

Nobel Prize was awarded to Jerne not for any particular laboratory research, but for his powerful, bold, far-reaching network theory of immune system that concerned the spatial relationship of antigen receptors and antibody idiotypes and their roles in regulating the immune response[5]. The theory was based upon a number of observations in the literature pertaining to the occurrence of idiotypes and on specific properties of the immune system. While theoretical contributions in physics, chemistry and mathematics had been frequently so acclaimed, this prestige was firstly extended to biomedical science with Jerne's principle on immune network in 1984.

Data from a large number of experiments support Jerne's general network concepts of immune system. Nevertheless, it has been recently envisaged among immunologists that the hypothesis can be more accountable and manageable with minor modifications[3]. Firstly, the immune responses induced through idiotypic immunizations are not open-ended as originally

conceived, but composed of multiple mini-networks. Ab_2 induced by idiotype-positive Ab_1 elicits Ab_3. Being similar to Ab_1, Ab_3 interacts with and neutralises the effects of Ab_2. Thus, the response seldom proceeds beyond Ab_3 physiologically. Secondly, all idiotypes are not necessarily networked in an immune regulatory cascade. This is supported by the recognition that only a subset of germline-encoded, cross-reactive idiotypes acts as regulatory idiotypes. Thirdly, idiotypic connectivity is not confined to B cells. Idiotypes have been recently demonstrated in TcRs, and the sharing of regulatory idiotypes between immunoglobulins and TcRs is likely[6,7]. Some TcRs can even directly interact with each other through shared idiotypic networks, though it is hard to be convinced unless a TcR idiotype is presented to an anti-idiotypic TcR as a processed linear peptide on a MHC molecule[8].

An antibody or immune receptor has 2 distinct properties as far as its idiosyncrasy is concerned: antigen-binding activity and idiotype. This is conceivable that antibodies directed against the paratope can display molecular mimicry of the original antigen. The possibility that these 2 properties of specific immune receptors may be related has led to numerous attempts to modify immune responses to specific antigens through the use of anti-idiotypes[9,10]. In the induction of the immune response to transplantation tolerance, idiotypic manipulation has been particularly appealing. Decrease of specific immune response has been found in separate experimental models[11,12,13].

1.2. Anti-idiotype Classification

Anti-Ids are immunochemically heterogeneous. They can recognize idiotypes either associated with the complementarity determining regions or with the framework portions of the variable region of antibodies or antigen receptors (Figure 1.3). When the binding of Ab_2 to idiotype-positive Ab_1 is associated with the hypervariable segments, the binding is inhibited by the specific antigen or hapten[14]. On the other hand, when Ab_2 binds to idiotopes which are located on the framework regions, the binding is not antigen or hapten inhibitable[15]. Furthermore, the ability of anti-Ids to elicit an antibody response is an other major characteristic for the taxonomy of Ab_2.

Based upon their immunochemical and functional idiosyncrasies, anti-Ids are originally assorted into 2 categories, namely: $Ab_{2\alpha}$ and $Ab_{2\beta}$[16]. The classification is subsequently expanded further with the addition of 2 more new categories; $Ab_{2\gamma}$ and $Ab_{2\epsilon}$[17].

$Ab_{2\alpha}$ are antibodies which bind to the idiotype of the antigen receptor of B or T cells through their paratope - complementarity-determining region formed by the heavy and light chains. $Ab_{2\alpha}$ can also bind to the paratope-related idiotype outside the antigen combining site of Ab_1. These two categories of antibodies are specific for framework-associated idiotypes. The apparently non-competitive binding of Ab_2 to idiotype-positive Ab_1 can induce

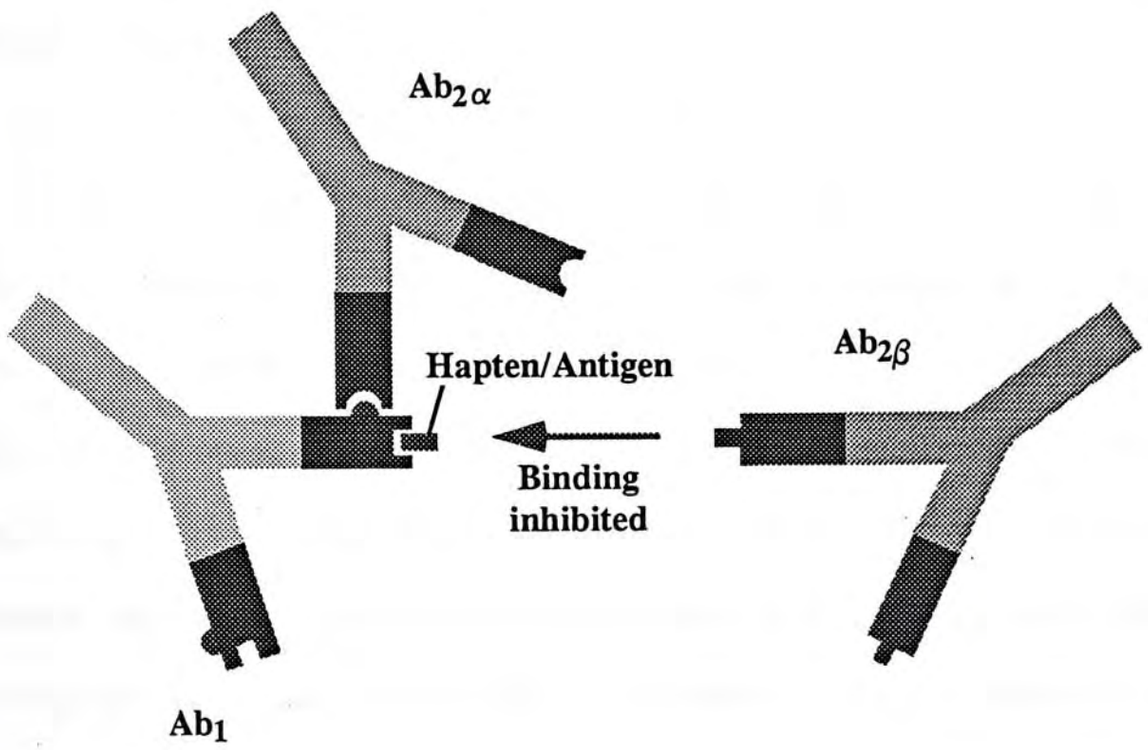


Figure 1.3. Conceptual distinction between $Ab_{2\alpha}$ and $Ab_{2\beta}$.

conformational changes of Ab_1. Eventually the equilibrium constant and the affinity of Ab_1 to specific antigen or hapten can also be altered.

$Ab_{2\gamma}$ is, in fact, a subcategory of the $Ab_{2\alpha}$. The idiotype to which $Ab_{2\gamma}$ binds is so close to the Ab_1 paratope that it interferes with nominal antigen or hapten binding. In an other word, $Ab_{2\gamma}$ is antigen- or hapten-inhibitive. It is recognized solely for experimental reasons to distinguish the so-called internal image of a special $Ab_{2\alpha}$[17].

$Ab_{2\beta}$ is combining site associated. Its idiotype fits well as an antigen in the Ab_1 paratope of antibodies, and B or T cells. Paradoxically $Ab_{2\beta}$ may imitate the original epitope and contribute to the internal image. Antibodies bearing an idiotypic determinant which mimics the 3-dimensional structure of the antigen are also known as homobodies[18]. These antibodies can mimic foreign antigens borne by viruses, parasites, protozoa and many other biologically active metabolites such as hormones[17]. Like antigens or bio-metabolites, $Ab_{2\beta}$ compete for binding to the antigen receptors of lymphocytes and other somatic cells.

$Ab_{2\epsilon}$ (epibodies) are capable to react with an antigen and with an idiotype of an antibody towards the same antigen[19,20]. The peculiarity of $Ab_{2\epsilon}$ was believed to be mimicry of the amino sequences and/or the 3-dimensional structures shared by an antigen and an idiotype of an antibody towards the

same antigen. They are originally described in a study which aimed at defining the idiotype of murine antibodies specific for the major cross-reactive idiotype of human IgM paraproteins exhibiting rheumatoid factor activity. An epibody can represent a link of connectivity between various mini-networks specific for foreign antigens and auto-antigens, a kind of "superantibody"[21].

1.3. Blood Transfusion Effect

Kidney transplantation is the most important and effective therapy for patients suffering from end-stage renal failure. Such patients are inevitably anaemic, and blood transfusion is quite often necessary prior to prospective renal transplantation, and apparently serves as an indispensable supplement to haemodialysis as well as an empirical subsidy to patient's well being.

The effect of blood transfusion effect on renal transplantation has been controversial and confusing. The presence of lympho-cytotoxic antibodies against donor in the pre-transplant serum of the recipient with hyperacute renal rejection was suggested to be induced by blood transfusion[22]. However, about a decade later it was observed that patients who had not received a pre-transplant blood transfusion had poor renal graft survival and predisposed to subacute rejections[23].

Following Opelz and his co-workers' striking report, the beneficial effect of pre-transplant blood transfusion on graft survival was also subsequently shown in animal models which included monkeys[24], dogs[25,26], mice[27] and rats[28]. These observations led to a liberal and sometimes deliberate transfusion policy, which contributed to the improvement of renal transplant outcomes till the early 1980s.

In 1983, this beneficial effect of blood transfusion effect was overshadowed by the introduction of cyclosporin[29]. Also in 2 multicenter studies separately conducted by the Scandinavian group[30] and the Collaborative Transplant Study[31], this transfusion effect appeared to have lost most of its importance in renal transplants performed in 1984 and 1985. On the contrary, the beneficial blood transfusion effect was consistently demonstrated in the Transplant Registry of the University of California at Los Angeles from 1980 to 1987[32].

1.4. Transfusion Protocol

Though most experts are virtually convinced by the fact that pre-transplant transfusion improves renal transplant survival, there is no unanimity among them as to what the best transfusion modality is.

One school of thought was headed by Opelz, who was the first to document clearly the beneficial pre-transplant transfusion effect[23]. He advocated at least 5 pre-transplant blood transfusions and they should be spaced at 2 to 3-month intervals. However, others contended that a single blood transfusion was enough and the effect was very long lasting[33,34].

The scenario became even more complicated as the transfusion effect could also be shown in recipients who had received deliberate donor-specific transfusions[35]. Patients administered approximately 300 mL donor-specific whole blood, on three separate occasions at 2-week intervals, had comparable graft survival to those who received kidneys from HLA-identical sibling donors.

An analysis of data collected during the South Eastern Organ Procurement Foundation Prospective Study from 1977-1982 identified the relative effects of different blood products on graft survival in cadaveric donor renal transplant recipients[36]. A significant increase in actuarial graft survival was seen in primary recipients who had pre-transplant transfusions with only frozen blood, washed blood, packed blood, or any combination of blood products as compared with those who received no transfusion. No blood product was found to provide a significantly greater increase in graft survival than any other blood product. The same was also found in re-grafted patients.

On the other hand, several studies reported excellent results in enhancing

renal graft survival by employing a policy of random (third party) blood transfusion[37,38,39]. Interestingly 2 recent reports show that the transfusion effect could only be detected in HLA-DR mismatched transplant recipients but not in HLA-DR-compatible transplants[32,40]. This finding implies that HLA-DR matching masks the transfusion effect.

There is also elusive and controversial evidence upon the timing of blood transfusion in acquiring the optimal effect. No significant difference is observed whether blood transfusions are given several months or just a few days prior to renal transplantation[41,42]. Available data indicate that per-operative blood transfusions are also effective[43], though this could not be confirmed in 2 large series of patients[44,45], and per-operative transfusion merely exerts an effect intermediate between pre-transplant transfusion and no transfusion at all[46]. Moreover, a significant improvement was obtained by per-operative transfusions for re-grafts[47].

The type of blood product responsible for producing the transfusion effect has not been fully elucidated, but nevertheless leucocytes are critical in achieving the beneficial effect. Both fresh blood and buffy coat are feasible[48,49,50]. Platelets which only express HLA class 1 antigens was reported to be as effective as whole blood in improving renal graft survival in primates[51,52]. In man, however, platelet transfusions were not shown to achieve the effect and even produced a detrimental outcome[53,54].

1.5. Mechanism of the Beneficial Transfusion Effect

Despite the overwhelming data indicating the beneficial effect of blood transfusion in renal transplantation since 1973 and the general acceptance of liberal blood transfusion as a mean of immunological manipulation in renal transplantation, little is known about the underlying mechanism. Even after nearly 2 decades of investigation, the mechanism(s) has not yet been thoroughly clarified. The immunology concerned still remains as a phenomenology - an ever accumulating catalogue of phenomena and observations. Several hypotheses have been proposed; however, each of them probably works only in the certain particular experimental setting to account for the observed clinical effects. They include: donor selection, clonal deletion, suppressor cells induction, prostaglandins mediation, mixed chimerism motivation, Fc-receptor blocking antibodies stimulation and anti-idiotypic antibodies instigation.

1.5.1. Donor Selection

It has been postulated that the transfusion effect is just an artefact caused by the exclusion of antibody-producing responders. Approximately one third of multi-transfused prospective renal graft recipients become hyper-immunized and sensitized against the presumed donor and cannot be transplanted[55]. Thus, blood transfusion leads to the selection of patients who

cannot make donor-specific lympho-cytotoxic antibodies.

However, it is inconsistent with the observation of excellent graft survival in patients with increased mixed lymphocyte culture responses after donor-specific transfusion, whom should be regarded as high responders[56]. Moreover, the concomitant use of donor-specific transfusion and immunosuppressive regime results in a marked reduction in donor-specific sensitization, and the high transplant success rate , under these circumstances, is similar to that of non-sensitized patients[57]. A further argument against the selection of non-responders is the very low risk of developing broadly reactive antibodies after random blood transfusion[58]. Finally, improvement in graft survival after transfusion has been demonstrated even in HLA-identical recipient-donor pairs, to whom the donor selection hypothesis simply plays no role[55].

1.5.2. Clonal Deletion

It has been suggested[59] that the primary function of transfusion is presumably to immunize patients and not to induce tolerance. Blood transfusion can be modelled as a priming event. When antigens on donor blood cells are in contact with recipient's specific antigen receptors of T or B cells, they will trigger clonal proliferation and activation. After transplant, the graft causes a re-stimulation and an anamnestic response with accelerated and

vigorous cell divisions. The concomitant high-dose massive immunosuppression at the time of transplant eliminates these rapidly proliferating specific reactive cell clones and leads to self tolerance. Time is probably important for removing rejection-causing clones but leaving suppressive ones intact, yet their relative proportions are the key in improving graft survivals.

Immunosuppressive regime is mandatory in achieving the salutary transfusion effect. If immunosuppression was omitted, blood transfusion results in an accelerated rejection. Thus, transfusion or immunosuppression alone is ineffective. Transfusion effect materializes only with the synergy of immunosuppression[60].

However, this hypothesis cannot account for the evidence found in animal models, in which intravenous injection of allogeneic lymphocytes in non-immunosuppressed recipients has resulted in prolongation of allograft survival[61]. In addition, blood transfusions have not shown changes in cellular immune responses in man[62]. In experimental animal models, blood transfusion does not always elicit antibody production, and prolonged graft survival has been noted in recipients without the presence of any significant antibody[63,64]. Moreover, blood transfusion does not always trigger allo-immunization.

1.5.3. Suppressor Cells Induction

It has been well documented that one of the major functions of suppressor cells is to keep surveillance upon B and certain T lymphocytes, and hence indirectly controls and regulates antibody production and cellular functions respectively.

There is good indication to suggest that blood transfusion can possibly mediate active immunologic unresponsiveness to donor's allo-antigens by the induction of either specific or non-specific suppressor cells[65-69]. So far, most suppressor cells are T cells of either CD4-positive or CD8-positive subset, though some can also be prostaglandin-producing macrophages with the immunosuppressive reactivity[70,71].

However, there are still no firm data showing that prolongation of allograft survival is the result of suppressor cells which inactivate reactive T cells. In addition, there are 2 related problems with the study of suppressor cells. The first one is the limited access to or the absence of distinctive membrane markers. Due to the heterogenicity of suppressor cells, a good specific marker is at present virtually insurmountable without any unlikely contamination. The second problem is the difficulty in developing a reliable assay system. The most commonly used strategies in assay systems are *in-vivo* adoptive transfer investigations and *in-vitro* cell-mixing analysis. Due to ethical

consideration, *in-vivo* adoptive transfer studies which inevitably require certain extent of immunosuppression, such as low dose irradiation or chemotherapy can only be applied in animal models. On the other hand, *in-vitro* analysis of suppressor cells is particularly prone to artefactual errors such as lymphokine consumption or cytotoxic response by activated T cells[72].

T-cell mediated suppression is merely determined by the way antigen is presented to the immune system. A subset of T cell activated earlier can exert a different immune response from the same T cell subset activated at a later stage. The order in producing different lymphokines during T-cell activation may govern whether the result is suppression or responsiveness.

1.5.4. Prostaglandins Mediation

Prostaglandin E, the prostanoid derivatives of arachidonic acid, is a product of the cyclo-oxygenase pathway and is known to have multiple immunosuppressive properties, including inhibition of lymphocyte mitogenesis and interleukin-2 production. Short-lived, antigen non-specific, suppressor T cell generation has also been attributed to high level of prostaglandin E[73]. Yet non-specific transfusion-induced immunosuppressive mediators are thought to account for the beneficial effect of transfusion in prolongation of renal allograft survival[71,74,75,76].

In animal model, significantly increased prostaglandin E synthesis in peritoneal macrophage after allogeneic blood transfusion suggests an immunosuppressive effect of the blood transfusion-prostaglandin interaction[77]. In man, a dose-dependent increase of prostaglandin synthesis after transfusion is also noted[78].

On the other hand, it is crystal clear that the nutritional variations of dietary lipids do indeed change prostaglandin metabolism and may have a significant effect on the immune system. Lipid substrate may eventually be metabolized through the lipoxygenase pathway to thromboxane and the leukotrienes, which have prodigiously stimulatory effects and outweigh the immunosuppressive activities of prostaglandin[79].

1.5.5. Mixed Chimerism Motivation

The peripheral stem cells, though present in a small number in the transfused blood, can under certain conditions, dwell themselves in particular caches, induce low grade mixed allogeneic chimerism and promote immunologic tolerance[80,81].

The induction of low grade mixed chimerism may be influenced by the HLA disparity between donor and recipient. The lesser the HLA disparity, the

higher the chance for stem cells to implant themselves in privileged sites. They, together with their progeny, may be protected from immune attack by a veto-like mechanism[82,83,84]. Though the probability is extremely low, this process can be enhanced by T-cell depletion with monoclonal antibodies or mild pharmaceutical immunosuppression of the recipient.

In animal model, mixed chimerism can be induced[85,86,87]. Sharabi and Sachs demonstrated stable allograft survival in mice across a complete H-2 disparity by treatment with anti-CD4 + anti-CD8 monoclonal antibodies and low dose whole body irradiation followed by donor bone marrow transfusion[85]. Qin and co-workers described skin allograft tolerance by administration of anti-CD4 + anti-CD8 monoclonal antibodies in combination with donor bone marrow[86]. Mayumi and Good also showed that skin allograft tolerance in mice across H-2 and non-H-2 barriers could be induced by anti-Thy-1 treatment and donor bone marrow followed by cyclophosphamide[87].

This hypothetical explanation to the beneficial blood transfusion effect is not without challenge. It has been argued that the tolerance may be due to the artefact of effective clonal deletion or anergy of immunocompetent CD4-positive cells to prevent detrimental rejection responses. Furthermore, it has been documented that the combination of T-cell purging by anti-T cell monoclonal antibodies and allogeneic bone marrow infusion has failed to induce chimerism or tolerance, but has resulted in sensitizing the recipient instead[88].

1.5.6. Fc-receptor Blocking Antibodies Stimulation

The interaction of antibody-antigen complexes with cells of the immune system results in a wide array of responses ranging from effector functions such as antibody dependent cytotoxicity, mast cell degranulation and phagocytosis, to immuno-modulatory signals such as regulating lymphocyte proliferation and antibody secretion. All these interactions are initiated through the binding of the Fc-domain of antibodies or immune complexes to specialized cell surface receptors on haemopoietic cells such as monocytes and macrophages.

Fc-receptor is a signal transducing molecule and is one of the paramount and indispensable domains essential for the processing of immune complexes within the immunity system. Fc-receptor blockage will hinder immune complexes manipulation and, in turn, lead to immuno-compromisation and immunologic tolerance eventually[89].

The presence of humoral cytotoxic antibodies to the allograft recipient's lymphocytes has been reported prior to transplantation; however, some have been shown to be harmless[90]. They react with both B and T cells from a panel of normal donors or, in some cases, with B lymphocytes alone, but not with lymphocytes from patients with chronic lymphocytic leukaemia[91]. This finding indicates that the cytotoxic antibodies are not directed against HLA antigens, since cells from those leukaemic patients possess such antigens on their surfaces.

Non-cytotoxic allo-antibodies that presumably block Fc-receptor sites on B lymphocytes and macrophages following blood transfusions are demonstrated in prospective transplant patients with improved allograft survival[92,93,94] and during pregnancy[95]. Fc-receptor blocking antibodies are also detected in long-term survivors of renal allografts[96].

On the contrary, an overall decrease of graft survival in the presence of B lymphocyte antibodies has been reported[97]. The development of donor specific B lymphocyte antibodies after renal transplantation also casts much doubt upon this hypothesis[98,99].

1.5.7. Anti-idiotypic Antibodies Instigation

Theoretically, the existence of an anti-idiotypic network provides an exquisite way to dampen immune responses[100]. The formation of anti-idiotypes (Ab_2) directed against the variable regions of antibody combining sites or antigen receptors of T lymphocytes (Ab_1) is frequently considered as an important explanation for the beneficial transfusion effect in allograft tolerance. The clonal expansion of donor-reactive T cells in response to donor blood elicits a compensatory proliferation of anti-idiotypic B cells which produce antibodies that block the antigen-specific T-cell receptor or circulating antigen-specific antibodies at the time of engraftment. Donor-reactive T cells are, in fact, not

completely eliminated by post-transplant immunosuppression, but instead are held in check by the anti-Ids.

Certain data suggest that anti-idiotypic antibodies directed against antigen recognition sites on T-cells in human can be induced by blood transfusion[101]. Anti-Ids developed during the immediate post-transplant period in hepatic transplantation are capable to immuno-precipitate T-cell receptor[102].

In addition, the presence of anti-Fab antibodies and significantly higher anti-$F(ab)_{2\gamma}$ antibodies in pre-transplant sera are shown to correlate with better graft survival[103,104]. The development of anti-Ids directed against anti-HLA antibodies after donor-specific transfusions is consistent with the hypothesis that anti-Ids down-regulates allo-immunity[105].

However, it has been reported that anti-idiotypic antibodies enhance and potentiate, rather than inhibit, the Ab_1 activity[106,107,108]. In addition, immunologically mediated renal diseases can be modulated by interfering with the mechanism of production of autoantibodies of specific anti-idiotypic reactivity[109]. Furthermore, there are no studies which prove that anti-Ids can specifically prolong allograft survival.

1.6. Study Aims

One important goal of transplantation immunology is to make possible successful tissue or organ transplantation without long-term need of immunosuppression. Since 1973, blood transfusion has been appreciated as one of the simplest and most pragmatic ways to induce allograft tolerance in renal transplantation. Recently this beneficial effect of blood transfusion is being challenged, and doubts have been casted upon such practice.

The main theme of this project was to study the relationship between blood transfusion and the prevalence of anti-Ids against anti-HLA antibodies in serum samples of renal transplant recipients. A comparison was also made to the levels of anti-Ids in renal transplant patients and those found in a normal control group.

Moreover, quantitative comparison of anti-Ids was studied in the following categories: transfused renal transplant recipients versus a normal control group, transfused versus non-transfused patients with kidney transplant, and non-transfused renal transplant recipients versus a normal control group. Correlations of graft rejection and graft survival with transfusion were also examined.

Inference drawn from the findings of this project could shed some light

on whether blood transfusions were indeed beneficial to renal transplant recipients and should remain a viable therapeutical adjunct in these patients.

1.7. Technical Strategy

The techniques involved in this project were basically divided into 2 main sections. The first section primarily dealt with the purification and preparation of $F(ab')_2$ from Ab_1. The second portion employed ELISA to capture and measure Ab_2 in the serum samples (Figure 1.4).

Nine human polyclonal anti-HLA antisera which are specific and reactive to the most frequently expressed antigens of HLA-A, B and DR loci in the study patient population were used as the source of Ab_1. They were passed through chromatography columns packed with purified Protein A covalently coupled to agarose. Each purified Ab_1 after elution was then dialyzed and pepsinized to generate $F(ab')_2$. The yield was passed through the Protein-A column to remove the pFc' contamination. The affinity purified $F(ab')_2$ was further dialyzed to remove any residual unbound pFc' and extensively digested small fragments to eliminate any undesirable interference in the subsequent assay.

A method based upon the ELISA principle was then utilized to evaluate Ab_2 in serum samples. $F(ab')_2$ from Ab_1 was employed as the ligand to coat

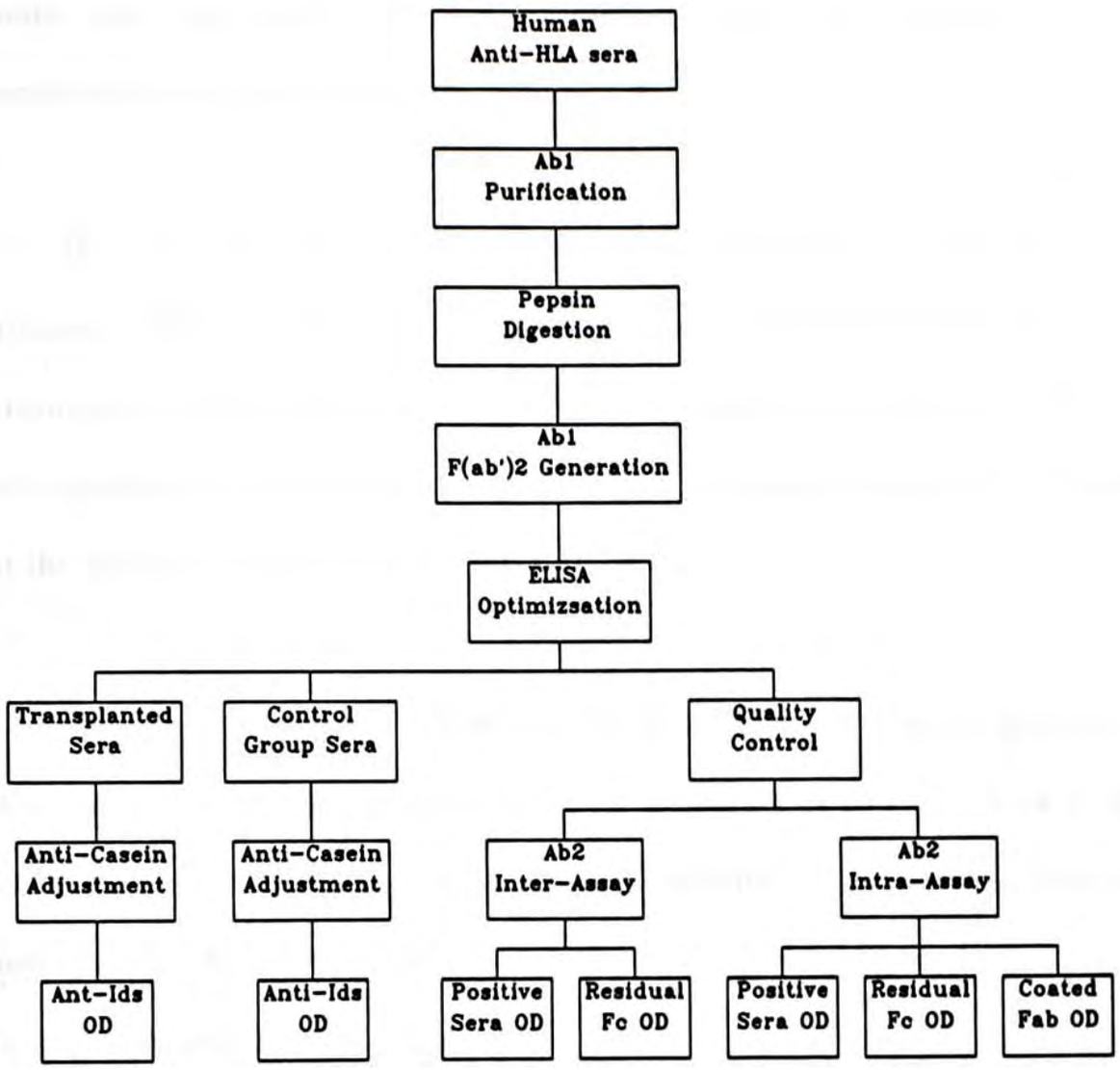


Figure 1.4. Flow chart showing the technical procedure involved in measuring anti-idiotypic antibodies against anti-HLA antibodies.

microplate and casein was utilized to inhibit non-specific bindings (see Method section 2.2.4). Prospective Ab_2 in the serum samples bound to Ab_1 $F(ab')_2$ were detected by Fc-specific, horse-radish peroxidase conjugated goat anti-human IgG and read spectrophotometrically with the addition of o-phenylenediamine dihydrochloride (Figure 1.5).

In order to make the analysis valid, a checkerboard titration was performed with a serum sample known to have a high anti-$F(ab')_2$ activity. Optimization of the study was resolved for the amount of coating $F(ab')_2$, nature and concentration of the blocking casein, dilution of the analyzing serum sample and the detector antibody (see Chapter 4).

Ab_2 against 9 Ab_1 were studied in 151 serum samples from 101 patients. Since temporal study of Ab_2 was possible for a number of patients, those with highest Ab_2 were enrolled into the Ab_2 kinetic study and statistical manipulation. Moreover, potential interference due to anti-casein was also offset (see Chapter 6).

Due to the non-availability of appropriate Ab_2 standards rendering the absolute quantitation impossible, strict quality controls on the inter-assay and intra-assay precision were carried out throughout the study to guard against any significant fluctuation of OD readings due to technical variables, and thereby validated the measurement of anti-Ids.

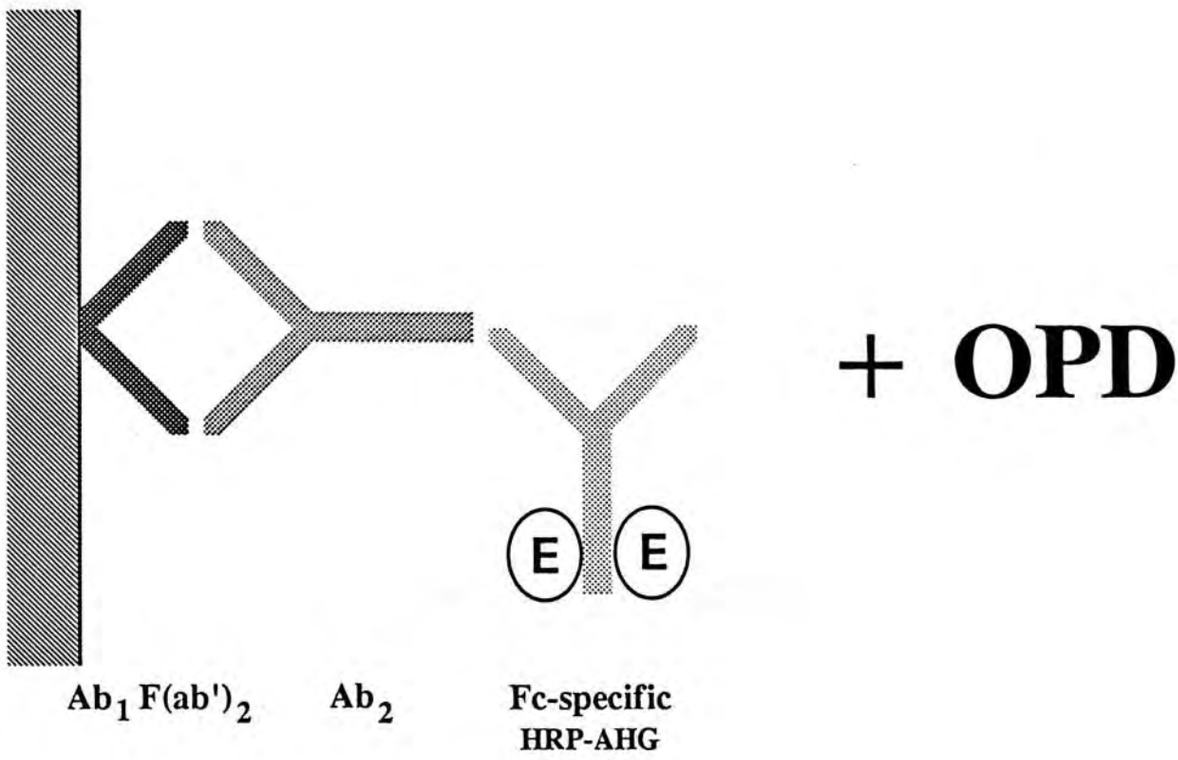


Figure 1.5. Diagramatic presentation of ELISA.

Chapter 2

Materials and Methods

2. Materials and Methods

2.1. Materials

2.1.1. Patient Population

Totally 101 ethnic Chinese patients in Hong Kong were recruited in this study with 48 having received cadaveric donor kidney and 53 transplanted living donor renal allografts. All grafts were ABO-compatible. Patient history such as sex and age, date of transplant, ABO and HLA typings and compatibilities, allo-immunization, graft rejection episodes, immunosuppressive drugs taken, renal biopsies, date and number of blood/blood products transfused, and any graft failure were all retrieved retrospectively. None of the patients had agammaglobulinaemia or paraproteinaemia.

2.1.2. Normal Control Group

Thirty-one apparently healthy subjects, 13 to 50 years old, without any history of organ transplant or blood transfusion, were recruited as the normal control group. The females in the control panel were nulliparous with no abortion history.

2.1.3. Serum Samples

Pre-transplant and post-transplant venous blood samples were drawn at weekly intervals. Venous blood samples were also collected from the normal control group. Sera were saved, dispensed into aliquots and stored at -70°C till tested.

2.1.4. Additional Specimens

Two different serum samples from patients suffering from systemic lupus erythematosus known to have anti-$F(ab')_2$ reactivity which were gifts from Mr. Albert Chan, Senior Research Technician of Renal Laboratory, under the auspices of Dr. Ignatius K.P. Cheng, Senior Lecturer of Department of Medicine, University of Hong Kong, were incorporated as positive controls in this study. One was employed in the optimization of the ELISA system while the other was used as an intra-assay and inter-assay positive control.

2.1.5. Chemicals

The following chemicals were obtained from Sigma, St. Louis, MO 63178, USA :

- Bovine serum albumin
- Crystallized and lyophilized Pepsin with 2500 - 3500 units per mg protein
- Carbonate buffer capsules of 0.05 M, pH 9.6
- p-Nitrophenyl phosphate tablets of 5 mg each
- Phosphate-citrate buffer capsules with 0.012% sodium perborate of 0.05 M at pH 5.0
- o-Phenylenediamine dihydrochloride tablets of 30 mg
- Monolaurate (Tween 20)
- Tris (hydroxymethylaminomethane)

The following were obtained from BDH, Dorset BH12 4NH, England :

- Casein soluble light white
- Disodium hydrogen phosphate
- Glycine
- Sodium acetate
- Sodium chloride
- Sodium dihydrogen phosphate
- Sodium hydroxide

The following were from Peking Chemical Works, Peking, PRC :

- **Acetic acid**
- **Hydrochloric acid**
- **Magnesium chloride**
- **Sulphuric acid**

2.1.6. Antisera

The following lyophilized human polyclonal antisera were from Hoechst-Behring, Marburg D-3550, Germany :

- anti-HLA-A11	Lot # 021108C
- anti-HLA-A24	Lot # 022401M
- anti-HLA-A2+A28	Lot # 028105C
- anti-HLA-B17	Lot # 021710C
- anti-HLA-Bw22+B7	Lot # 028404B
- anti-HLA-B40+B13	Lot # 028803A
- anti-HLA-DR2	Lot # 070205E
- anti-HLA-DR4	Lot # 070406G
- anti-HLA-DR7	Lot # 070706K

Affinity purified lyophilized goat anti-human IgG of 0.9 mg/mL, Lot # 061891J7, which is Fc-specific and conjugated with horse-radish peroxidase with suggested working dilution from 1:5000 to 1:100,000 for enzyme immunoassay was from Chemicon, Temecula, CA 92390, USA.

Affinity isolated goat anti-human IgG, Lot # 59F4813, which is Fab-specific and conjugated with alkaline phosphatase with suggested minimum working dilution 1:1000 was from Sigma, St. Louis, MO 63178, USA.

2.1.7. Buffers

All solutions were prepared from deionized and distilled water with 'Analar' grade chemicals :

Acetate Buffer, 20 mM pH 4.0

CH_3COONa	1.64 g/L
CH_3COOH	1.20 g/L

One volume CH_3COONa solution was mixed with 2 volumes of CH_3COOH solution till pH 4.0.

Carbonate Buffer, 50 mM pH 9.6

One capsule of carbonate buffer (Sigma, USA) was dissolved in 100 mL deionized and distilled water.

Carbonate Buffer with 5 mM $MgCl_2$, 25 mM pH 9.6

One capsule of carbonate-bicarbonate buffer (Sigma, USA) and 0.13 g $MgCl_2.2H_2O$ were dissolved in 200 mL deionized and distilled water.

Glycine-HCl Buffer, 20 mM pH 2.5

Glycine	1.50 g/L

pH adjusted to 2.5 with 0.02 M HCl

Phosphate Buffer Saline (PBS), 0.2 M pH 7.4

K_2HPO_4	26.60 g/L
KH_2PO_4	8.00 g/L
NaCl	8.77 g/L

pH titrated to 7.4 with 1 M NaOH or HCl

PBS, 10 mM pH 7.4

K_2HPO_4	1.33 g/L
KH_2PO_4	0.40 g/L
NaCl	8.77 g/L

pH adjusted to 7.4 with 1 M NaOH or HCl

Phosphate Buffer Saline-Tween (PBST)

PBS 10 mM pH 7.4	1 L
Tween-20	0.5 mL

Phosphate Buffer Saline-Casein (0.5% PBSC)

PBS 10 mM pH 7.4	1 L
Casein	5 g

pH adjusted to 7.4 with 5 M NaOH

Phosphate Buffer Saline-Casein (2.5% PBSC)

PBS 10 mM pH 7.4	1 L
Casein	25 g

pH adjusted to 7.4 with 5 M NaOH

Phosphate-Citrate Buffer with 0.012% Sodium Perborate, 50 mM pH 5.0

One capsule of phosphate-citrate buffer (Sigma, USA) was dissolved in 100 mL deionized and distilled water.

Tris-HCl Buffer, 10 mM pH 8.0

Tris	**1.21 g/L**

pH adjusted to 8.0 with 1 M HCl

2.1.8. Consumables

Affinity columns of 2.5 mL immobilized Protein A covalently coupled to agarose with binding capacity of 12-15 mg human IgG per mL of gel were from Pierce, Rockford, IL 61105, USA.

Dialysis cellulose membrane, Model Spectra/Por 7, with MW cutoff 50,000, volume capacity 0.46 mL/cm, cylinder diameter 7.6 mm and flat width 12 mm was from Spectrum, L.A. 90054, USA.

Micro-concentrators, Centricon-30 of MW cutoff 30,000 were from Amicon, Beverly, MA 01915, USA. The IgG recovery in the retentate was 96% of the starting concentration.

Microtitre plates, Model Immulon 3, in an arrangement of 96 miniature polystyrene flat bottom wells of volume capacity of approximately 0.4 mL each were from Dynatech, Chantilly, VA 22021, USA.

Polypropylene pipette tips with good precision were from Bio-Rad, Richmond, CA 94802, USA.

2.1.9. Apparatus and Equipment

High-precision adjustable Gilson pipettors, Models P10, P20, P200 and P1000 ranging from 10 to 1000 uL for serum and reagent dispensing and sampling were from Gilson, Villiers-le-Bel 95400, France.

A multichannel motorized pipette, Model EDP-Plus M8 from Rainin, CA 94608, USA was utilized in multiple sampling and dispensing in the ELISA.

A compact bench-top hot air incubator with shaker, Model LT Biomax 500 (Life Technologies, Singapore) was employed in all incubations.

A programmable bench top centrifuge with a fixed-angle rotor, Model Labofuge 200, was from Heraeus Sepatech, Germany.

Quartz cuvette and a diode array spectrophotometer, Model 8452A from Hewlett Packard linked to an IBM compatible PC, Model Commodore PC 40-III, were used to read absorbance (OD). The data capture was carried out with Hewlett Packard software and hard copies were available with an Epson LX-800 dot matrix printer.

The following equipment were obtained from Dynatech, Chantilly, VA 22021, USA :

- programmable and automatic 96-channel AM56 Ultra Washer II completed with vacuum pressure unit, liquid and vacuum bottles, bottle holder and all necessary tubings and connections.
- programmable and automatic MR5000 microplate reader linked with an Epson LX-800 dot matrix printer.

2.2. Methods

2.2.1. Purification of Human Polyclonal Anti-HLA Antisera

2.2.1.1. Affinity Chromatography

Lyophilized human polyclonal anti-HLA antisera (Hoechst-Behring, Germany) from primiparous and multiparous females were reconstituted with 0.5 mL deionized and distilled water, then were allowed to pass through the column packed with purified Protein A covalently coupled to agarose (Pierce, USA) under unit gravity with an hourly flow rate of 18 mL. The column was washed with 3 mL 10 mM Tris buffer pH 8.0 and the effluent was recycled twice into the column to maximize the IgG capture. The column with bound IgG was washed with Tris buffer pH 8.0 till no residual protein contaminant was detected as monitored by the OD of the effluent at 280 nm. The column with bound IgG was eluted with 20 mM glycine buffer pH 2.5. The IgG eluate of 0.5 mL fraction was allowed to drop into equal volume of 0.2 M phosphate buffer pH 7.4 for immediate neutralization. Fractions with OD greater than 0.05 at 280 nm were pooled.

2.2.1.2. Dialysis

Purified IgG was dialyzed against at least 2000 x volume 10 mM PBS pH 7.4 at 4°C using dialyzing tubing (Spectrum, USA) of 50,000 MW cutoff for 24 hours with 4 changes of buffer and continuous stirring.

2.2.1.3. Concentration

Approximate 2 mL purified antibody was added to a micro-concentrator, Centricon-30 (Amicon, USA) of 30,000 MW cutoff and spun with a fixed-angle rotor centrifuge, Labofuge 200 (Heraeus Sepatech, Germany) at 3000 x *g* for 15 minutes. Filtrate was discarded. Micro-concentrator was then inverted and spun at 1000 x *g* for 2 minutes to transfer the concentrate into the retentate cup and saved for later use.

2.2.1.4. Quantitation

The final volume of the concentrated IgG was measured and the OD was read at 280 nm in a quartz cuvette against 10 mM PBS pH 7.4 as reagent blank using a spectrophotometer (Hewlett Packard, USA) linked to an IBM compatible PC (Commodore, USA) and a dot matrix printer (Epson, USA). The purified

quantity of anti-HLA antibody was computed with the coefficient of extinction of human IgG[110].

2.2.2. Generation of $F(ab')_2$ fragments from the Purified Human polyclonal anti-HLA antibodies

2.2.2.1. Buffer Exchange

Concentrated IgG in 10 mM PBS was dialyzed against 2000 x volume 20 mM sodium acetate buffer pH 4.0 at 4°C for 6 hours to have thorough buffer exchange.

2.2.2.2. Pepsin Digestion

The non-specific protease which is active only at acid pH and is irreversibly denatured at neutral or alkaline pH cleaves normally at the C-terminal side of the inter-heavy chain disulphide bonds and produces a bivalent antigen binding fragment, $F(ab')_2$, with MW about 105,000. The Fc portion undergoes extensive degradation into pFc' of MW 27,000 and small peptides.

Crystalline pepsin (Sigma, USA) was added to the purified IgG in 20 mM

acetate buffer pH 4.0 in a ratio of 1:50 (w/w) and incubated for 20 hours at 37°C. Digestion was stopped by adding 10 mM Tris buffer pH 8.0.

2.2.2.3. Purification of $F(ab')_2$

To remove the undigested IgG, pFc'-fragments and extensively digested small polypeptides, the preparation was run through a Protein A column (Pierce, USA) equilibrated with 10 mM PBS pH 7.4. Those Protein A-unbound fractions of $F(ab')_2$ of MW about 105,000, the likely contamination with pFc'-fragments of MW 27,000 and extensively small polypeptides, with OD greater than 0.05 were pooled, dialyzed against 10 mM PBS pH 7.4 in dialysis tubing of MW cutoff 50,000 and concentrated as stated in Method Sections 2.2.1.2. and 2.2.1.3. The yield was dispensed into aliquots and stored at -70°C for later use.

2.2.3. Enzyme-Linked Immunosorbent Assay for anti-Idiotypes against anti-HLA antibodies

2.2.3.1. Optimization

To ensure that the anti-idiotypes in test sera could be captured by an optimal amount of anti-HLA $F(ab')_2$, a checkerboard titration was performed

on 1 of the 2 serum samples known to have a high anti-$F(ab')_2$ activity (see Material section 2.1.4).

One hundred uL of 1, 2, 5, 10 to 20 ug/mL specific anti-HLA $F(ab')_2$ in 0.05 M carbonate buffer pH 9.6 were used to coat microtitre plates (Immulon 3, Dynatech Lab., USA) overnight at 4°C. The plates were then washed 10 times in 10 mM PBS pH 7.4 with 0.05% Tween (PBST). Non-specific binding was blocked with 200 uL of 2.5% casein (BDH Chemicals Ltd, England) in 10 mM PBS pH 7.4 (PBSC) for 1 hour at 37°C. After washing, 100 uL of 1:50 to 1:400 normal serum control and serum control with high anti-Ids in 0.5% PBSC were added in quadruple and incubated for 3 hours at 37°C. The plates were washed again 10 times with PBST. Hundred uL of 1:5000 to 1:40000 Fc-specific, horse-radish peroxidase conjugated goat anti-human IgG (Chemicon, USA) in 0.5% PBSC was added and incubated 2 hours at 37°C. Plates were subsequently washed 10 times with PBST and developed using 100 uL of 0.6 mg/mL o-phenylenediamine dihydrochloride in 50 mM phosphate-citrate pH 5.0 containing 0.012% sodium perborate (Sigma, USA) as a substitute for H_2O_2. The 7-minute reaction was stopped by adding 50 uL of 4 M H_2SO_4 and the orange-brown end-point was read spectrophotometrically with the Dynatech MR5000 microplate reader (Dynatech, USA) at 492 nm against the reagent blank; i.e. the OD generated from wells without the addition of test serum.

2.2.3.2. Quality Control

2.2.3.2.1. $F(ab')_2$ Specificity

One hundred uL of 2 ug/mL specific anti-HLA $F(ab')_2$ in 0.05 M carbonate buffer pH 9.6 was used to coat microtitre plates overnight at 4°C. The plates were then washed 10 times in PBST. Non-specific binding was blocked with 200 uL of 2.5% PBSC for 1 hour at 37°C. After washing, 100 uL of 1:2000 goat anti-human $F(ab')_2$ conjugated with alkaline phosphatase (Sigma, USA) in 0.5% PBSC was added and incubated for 2 hours at 37°C. The plates were then washed and developed using 100 uL of 1 mg/mL disodium p-nitrophenyl phosphate (Sigma, USA) in 25 mM bicarbonate buffer pH 9.6 with 5 mM $MgCl_2$. The reaction was stopped by adding 50 uL 0.75 M NaOH after 20 minutes. The yellow colour of nitrophenol was measured at 405 nm with the microplate reader against the reagent blank, i.e. the OD generated from wells without the coating of $F(ab')_2$.

2.2.3.2.2. Fc-fragments Contamination

The testing procedure for the likely contamination by the residual undialyzed Fc-fragments in the $F(ab')_2$ preparation was similar to that described in Method Section 2.2.3.2.1., except 100 uL of 1:30000 HRP-AHG was directly

added after blocking and OPD was used as the chromogenic substrate for colour development. The 7-minute reaction was stopped by the addition of H_2SO_4 and the OD was read.

2.2.3.2.3. Precision Test

To guard against the precision and reproducibility of the assay, positive serum controls with high anti-$F(ab')_2$ activity and reagent blanks in parallel with the test samples were performed within and between runs of the testing protocol. The data were compiled and C.V.s were calculated. Test results from batches that generated control OD values which fell out of 95% significance interval were discarded and the testing was repeated.

2.2.4. Anti-Casein Interference

Due to the prevalence of intolerance of milk and milk products intake from ethnic Chinese and the possibly formation of the corresponding nuisance anaphylactic antibodies, the possible interference to the anti-Ids assay caused by anti-casein was also under consideration.

Wells without the coating of anti-HLA $F(ab')_2$ were blocked with 200 uL

of 2.5% PBSC for 1 hour at 37°C, washed, and 100 uL of 1:100 test serum in 0.5% PBSC was added in duplicate and then subsequently processed in the same way as cited in Method section 2.2.3.2.2. The orange-brown end-point against the reagent blank yielded the OD caused by anti-casein in the test sera.

In order to minimize the possible interference, auto-control for anti-casein was run in parallel with test for Ab_2. Correction was made by subtracting the anti-casein background from that of test.

2.2.5. Test Protocol

The testing procedure was the same as stated in Method section 2.2.3.1., except with 100 uL of 2 ug/mL anti-HLA $F(ab')_2$ for plate coating, 100 uL of 1:100 for assaying serum samples and 100 uL of 1:30000 HRP-AHG for the abducted anti-Ids. Positive controls were run in parallel. All tests were performed in quadruple.

2.3. Statistical Analysis

The comparison involved 2 sets of separate, independent samples from assumed normal distributions, namely; renal transplant patients versus normal

subjects, transfused renal graft recipients versus non-transfused counterparts, and non-transfused transplant patients against normal subjects as well.

Z- test[111] was applied to manipulate the naturally unpaired data. For the spare data which the total degree of freedom was less than 50, the unpaired Student's *t*- test[112] was used. With data that revealed apparent difference in the variances of 2 naturally unpaired samples, *F*- test was utilized[112]. When the sample sizes were very small, Mann-Whitney U - Wilcoxon Rank Sum W Test was employed. The data matrices were also subjected to Chi-square analysis[111].

Chapter 3

Purification of

Anti-HLA IgG and $F(ab')_2$

3. Purification of Anti-HLA IgG and $F(ab')_2$

3.1. Immunoglobulin Concentration

Polyclonal anti-HLA antisera of known specificities from commercially available lyophilized human sera of primiparous and multiparous females were affinity-purified by passing through columns packed with purified Protein A covalently coupled to agarose. The respective anti-HLA IgG harvested from 0.5 mL lyophilized ampoules are shown in Table 3.1. The yield, ranging from 2.14 to 4.34 mg, was quantitated by means of the following equation and the human IgG extinction coefficient.

$$\text{Yield (mg/mL)} = A280 / EC \times DF$$

where A280 is the OD of yield at 280 nm,

EC is the human IgG extinction coefficient of 1.43, and

DF is the dilution factor.

3.2. $F(ab')_2$ Specificity

An absolute method for protein determination[113] based on the difference in OD values at 235 and 280 nm was adopted for the $F(ab')_2$ quantitation in this

Table 3.1. Harvest of anti-HLA IgG and $F(ab')_2$ from 0.5 mL lyophilized human anti-HLA antisera

	Yield (mg)	
Antibody Specificity	IgG	$F(ab')_2$
Anti-HLA-A11	3.42	1.00
Anti-HLA-A24	2.14	0.55
Anti-HLA-A2+A28	2.79	0.72
Anti-HLA-B17	3.18	1.25
Anti-HLA-Bw22+B7	4.34	1.77
Anti-HLA-B40+B13	3.08	0.82
Anti-HLA-DR2	3.63	1.10
Anti-HLA-DR4	2.79	0.79
Anti-HLA-DR7	2.71	0.72

project. The equation used is as follow:

$$\text{Protein concentration (mg/mL)} = (A235 - A280) / 2.51$$

where A235 and A280 are the OD values at 235 and 280 nm respectively.

The mean recovery of the digested product, $F(ab')_2$ after pepsinization is 30.29%; ranging from 25.70 to 40.78% (Table 3.1). The $F(ab')_2$ preparations of 9 purified Ab_1 were checked for any noticeably adverse over-degradation and possible loss of Fab immunochemical reactivity after digestion, and the results were clearly indicated in Table 3.2. The mean OD readings detected by alkaline phosphatase-conjugated, Fab-specific goat anti-human IgG on 9 Ab_1 $F(ab')_2$ preparations of 0.2 ug (2 ug/mL) were significantly higher than those of the reagent blank after a 20-minute incubation ($p \ll 0.01$). The Fab specificity was guaranteed.

3.3. Fc-fragments Contamination

As shown in Table 3.3, the mean OD readings generated by horse-radish peroxidase-conjugated, Fc-specific goat anti-human IgG on 9 Ab_1 $F(ab')_2$ preparations of 0.2 ug were extremely low; ranging from 0.041 to 0.063. The likely Protein A-unbound Fc-fragments and those extensively pepsin-digested but undialyzed small fragments were low in these 9 Ab_1 $F(ab')_2$ preparation.

Table 3.2. Mean absorbance of $F(ab')_2$ preparations detected by Fab-specific alkaline phosphatase-conjugated goat anti-human IgG

$F(ab')_2$ Specificity	n	OD	SD	SEM	%CV
Anti-HLA-A11	24	0.274	0.008	0.002	2.81
Anti-HLA-A24	24	0.253	0.006	0.001	2.20
Anti-HLA-A2+A28	24	0.275	0.006	0.001	2.09
Anti-HLA-B17	24	0.255	0.008	0.001	3.14
Anti-HLA-Bw22+B7	24	0.278	0.006	0.001	2.21
Anti-HLA-B40+B13	23	0.271	0.011	0.002	3.98
Anti-HLA-DR2	24	0.258	0.006	0.001	2.47
Anti-HLA-DR4	24	0.283	0.007	0.002	2.61
Anti-HLA-DR7	24	0.248	0.006	0.001	2.61
Reagent Blank	50	0.104	0.006	0.001	5.70

where :

n = number of tests
OD = Mean Absorbance
SD = Standard Deviation
SEM = Standard Error of Mean
%CV = Coefficient of Variation in percentage

Table 3.3. Mean absorbance of $F(ab')_2$ preparations detected by horseradish peroxidase-conjugated Fc-specific goat anti-human IgG

$F(ab')_2$ Specificity	n	OD	SD	SEM	%CV
Anti-HLA-A11	52	0.048	0.003	0.0005	7.12
Anti-HLA-A24	52	0.055	0.004	0.0006	7.23
Anti-HLA-A2+A28	54	0.041	0.005	0.0007	13.40
Anti-HLA-B17	63	0.048	0.005	0.0006	9.58
Anti-HLA-Bw22+B7	49	0.063	0.005	0.0008	8.70
Anti-HLA-B40+B13	52	0.046	0.003	0.0005	7.08
Anti-HLA-DR2	50	0.046	0.006	0.0008	12.20
Anti-HLA-DR4	38	0.056	0.005	0.0008	8.52
Anti-HLA-DR7	51	0.041	0.005	0.0007	11.40
Reagent Blank	50	0.044	0.006	0.0008	12.70

where :

n = number of tests
OD = Mean Absorbance
SD = Standard Deviation
SEM = Standard Error of Mean
%CV = Coefficient of Variation in percentage

3.4. Discussion

Even though the yield of IgG from 9 human anti-HLA antisera after affinity chromatography purification and the $F(ab')_2$ preparations after pepsin digestion were not quite high, they were adequate as a source of ligand in the subsequent ELISA.

The employment of 100 uL of 2 ug/mL $F(ab')_2$ preparations of 9 affinity-purified human polyclonal anti-HLA antibodies in microplate coating was checked being valid and efficiently suitable for anti-Ids detection. The Fab specificity was retained without any apparently noticeable deterioration of immunochemistry as shown by the goat anti-human Fab reactivity. Moreover, the background OD caused by the interference of the likely residual Fc-contamination in these working $F(ab')_2$ preparations were insignificant and within acceptable limits.

Chapter 4

ELISA Optimization

4. ELISA Optimization

4.1. Coating $F(ab')_2$ Quantitation

The checkerboard plot (Figure 4.1) shows the mean OD of quadruple analyses from titrations of 100 uL of 1, 2, 5, 10 to 20 ug/mL specific anti-HLA $F(ab')_2$ with Fc-specific HRP-conjugated goat anti-human IgG of dilutions from 1:5000 to 1:40000. OD value not greater than 0.05 was chosen as a set point for tolerably nominal background interference. High background OD readings were observed in over-coated microplates with anti-HLA $F(ab')_2$ preparations greater than 0.5 ug. $F(ab')_2$ preparations of 5 ug/mL or higher concentration were thus considered inappropriate for use.

The anti-HLA $F(ab')_2$ preparation of 1 ug/mL gave comparatively low OD with Fab-specific AP-AHG at manufacturer-recommended working concentration of 1:2000 (Table 4.1).

Anti-HLA $F(ab')_2$ preparation, at concentration of 2 ug/mL, which exhibited a characteristic of relatively higher anti-Fab binding (Table 4.1) with comparatively lower background interference (Figure 4.1) was considered the optimal coating concentration. It was eventually selected for microplate coating for all subsequently assays.

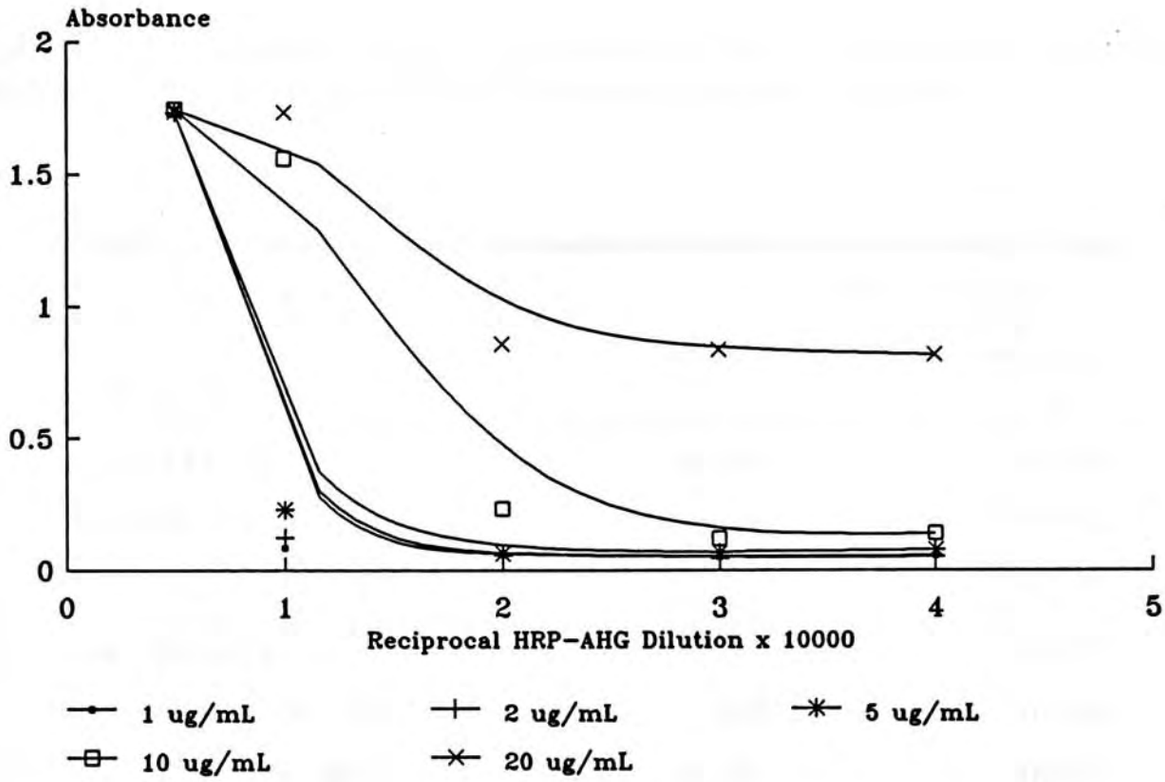


Figure 4.1. Interaction between $F(ab')_2$ coating concentration for ELISA wells and the working dilution of Fc-specific HRP-AHG.

Table 4.1. Comparison of mean absorbance of $F(ab')_2$ preparations detected by alkaline phosphatase-conjugated Fab-specific goat anti-human IgG.

	Concentration (ug/mL) of $F(ab')_2$ Preparation	
$F(ab')_2$ Specificity	1	2
HLA-A11	0.193	0.274
Anti-HLA-A24	0.181	0.253
Anti-HLA-A2+A28	0.192	0.275
Anti-HLA-B17	0.180	0.255
Anti-HLA-Bw22+B7	0.195	0.278
Anti-HLA-B40+B13	0.191	0.271
Anti-HLA-DR2	0.186	0.258
Anti-HLA-DR4	0.203	0.283
Anti-HLA-DR7	0.178	0.248
Reagent Blank	0.110	0.104

4.2. Blocking and Diluting Agent Concentration

The efficacy in the blocking of non-specific binding by 1% bovine serum albumin (BSA) was compared with 2.5% casein both in 200 uL PBS pH 7.4 (Figure 4.2). The mean OD of the OPD substrate at 492 nm, from quadruple wells, developed after 7-minute incubation by the non-specific binding of 100 uL HRP-AHG of dilutions from 1:20000 to 1:40000 in PBS pH 7.4, on plain microplates without any anti-HLA $F(ab')_2$ coating, and blocked with 2.5% PBSC at 37°C for 1 hour, was found more superior than that of 1% BSA under the same condition. Moreover, the non-specific binding was further decreased with the dilution of HRP-AHG being made in 0.5% PBSC. OD values around 0.05 were consistently obtained (Figure 4.2).

4.3. Serum Analyte Dilution

In order to elucidate the optimal working dilution for serum analyte, titrations from 1:50 to 1:400, of a serum sample known to have high anti-$F(ab')_2$ activity, were made against anti-HLA $F(ab')_2$ preparations of concentration 1, 2, 5, 10 and 20 ug/mL separately. Figure 4.3 shows the mean OD of quadruple tests detected with HRP-AHG conjugate at 1:30000.

Two-component curves were shown with a relatively rapid decline of OD

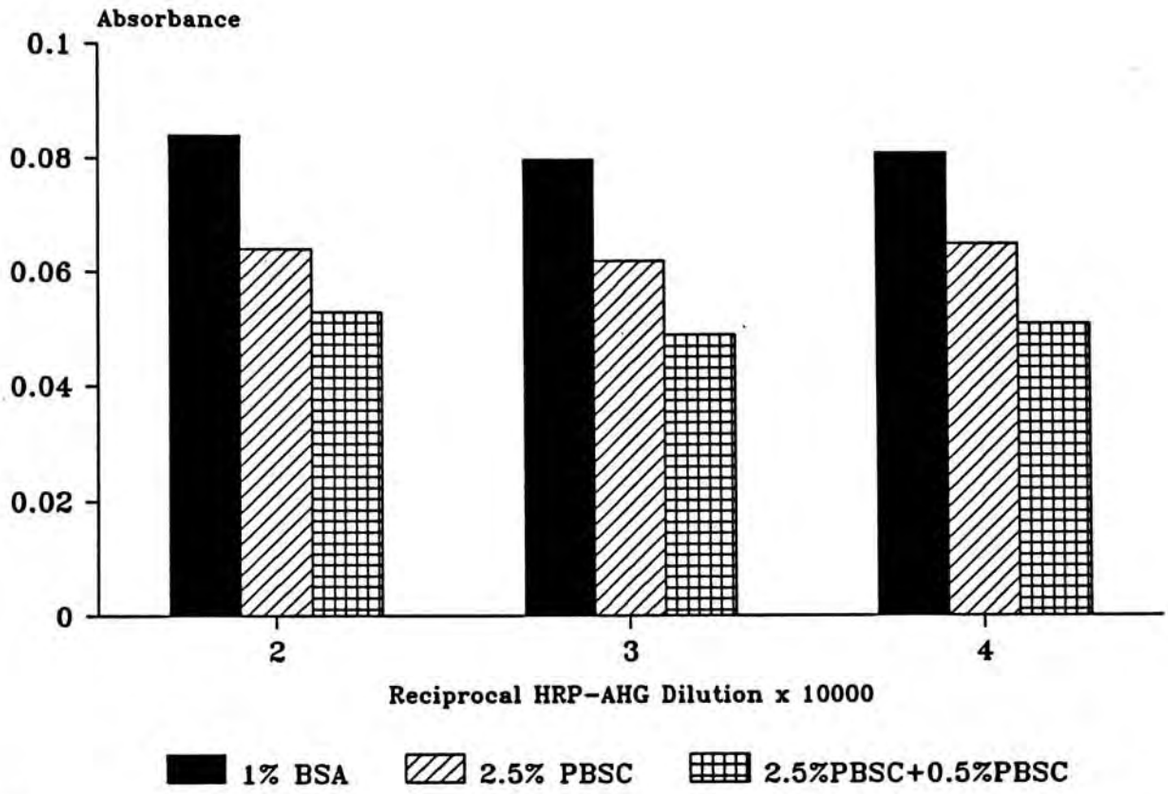


Figure 4.2. Comparison of the efficacy of 1% BSA and 2.5% PBSC in blocking non-specific binding. Further improvement was acquired with 0.5% PBSC being used as diluent.

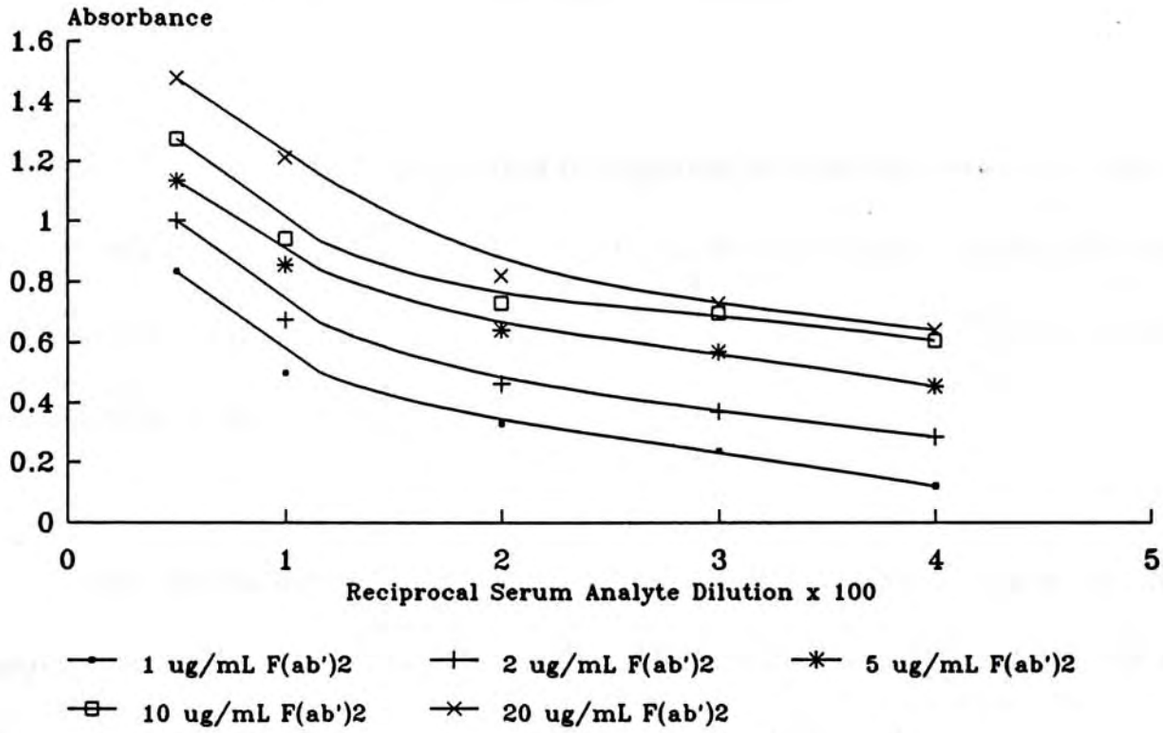


Figure 4.3. Titration of serum analyte against concentrations of anti-HLA $F(ab')_2$ preparations.

from serum dilutions of 1:50 to 1:100, then followed by a gradual flattening at 1:200 onwards. Dilution of serum analyte at 1:100 stood out to be the optimal one with all 5 anti-HLA $F(ab')_2$ preparations (Figure 4.3).

4.4. Conjugated Detector Antibody Titration

It has already been shown that the dilution of 1:30000 stood out to be the optimal working concentration of Fc-specific HRP-AHG in giving insignificantly low background interference with pre-determined levels of anti-HLA $F(ab')_2$ preparations (Figure 4.1).

The manufacturer's recommended working dilution range of the conjugated detector antibody is from 1:5000 to 100000. Evaluation of an optimal working dilution was performed with 1:100 of a serum sample previously known with high anti-$F(ab')_2$ activity (Figure 4.4). A 2-component curve, with a comparatively sharp decline at serum dilutions from 1:5000 to 1:25000, and eventually followed by a onward gentle flattening, was again noted.

The Fc-specific HRP-AHG at 1:30000 was established in this experimental setting as the optimal working dilution with respect to an acceptable background OD around 0.05 and the proficiency to detect

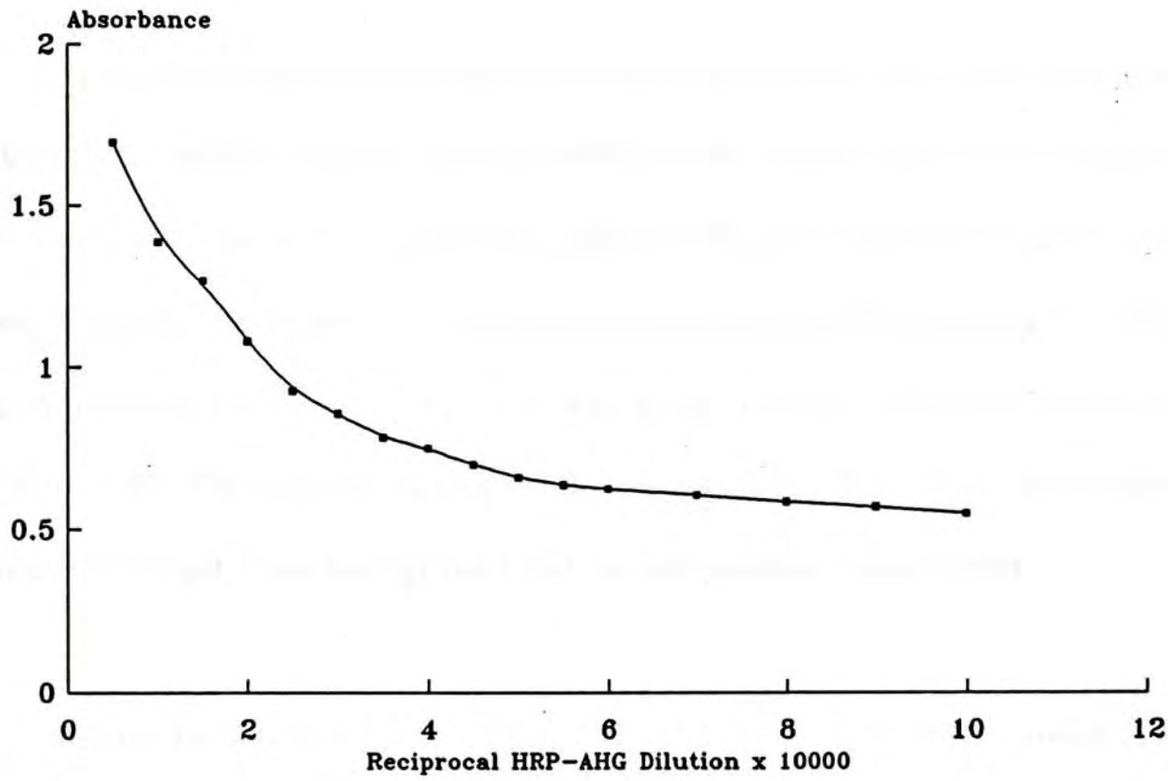


Figure 4.4. Titration of Fc-specific HRP-AHG against a serum of known anti-$F(ab')_2$ activity at 1:100 dilution in microplate wells coated with 0.2 ug anti-HLA $F(ab')_2$ preparation.

prospective Ab_2 in 1:100 dilution of serum analyte.

4.5. Discussion

Less than adequate coating of the microplate will reduce sensitivity and afford non-specific binding opportunities for the analyte and the conjugated detector antibody to the plastic microplate well and eventually leads to high backgrounds. However, over-coating induces non-specific trapping[114]. High signal-to-noise ratios are imperative for good sensitivity in all ELISA tests (Figure 4.5). The selected quantity of 0.2 ug anti-HLA $F(ab')_2$ for plate coating fulfils the target - low background but no compromised sensitivity!

Proteins do differ considerably in their ability to block non-specific binding. Being dispersed or solubilized in water to form micelles, amphipathic proteins, such as casein, which contain both strongly non-polar and mighty polar groups are especially effective in preventing non-specific binding[115]. The ability of casein in blocking non-specific binding may be attributed to the formation of a mono-molecular layer that prevents almost all other protein adsorption to the plastics.

The choice of blocking agent in an attempt to inhibit non-specific binding can be critical to the sensitivity and specificity of ELISA system[116]. Casein has

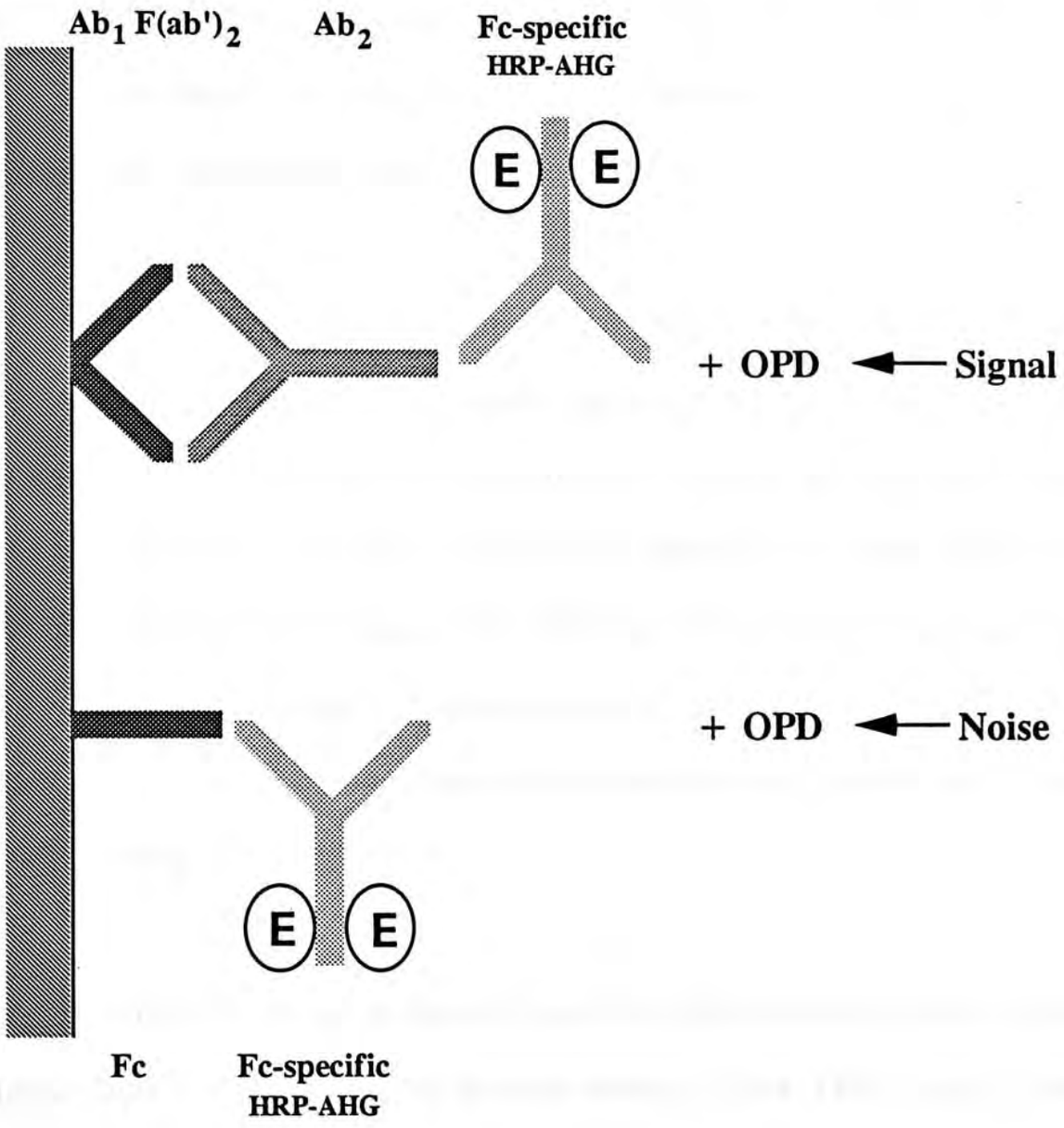


Figure 4.5. The likely residual Fc-fragments contamination in the ligand Ab_1 $F(ab')_2$ preparation may cause untoward noise generation. Optimization of the ELISA system to produce high signal-to-noise ratio is prerequisite.

been documented to be a more effective blocking agent than either BSA or gelatin[117]. Yet blocking proteins are largely chosen by convenience and empirical testing in specific ELISA settings. The blocking with 2.5% PBSC together with dilutions of serum analytes and conjugated antibody in 0.5% PBSC showed that there was apparently no room for the traditional employment of BSA in this particular experimental situation.

The optimal dilution of serum analyte should cover and give a clear differentiation of OD values with various degrees of Ab_2 positivity, be capable and sensitive enough to detect weakly positive samples and yet exhibit low background interference. Data in Figure 4.3 showed that serum dilution at 1:100 or lower, just below those higher dilutions which generated an apparent OD flattening, were preferred because of higher reactivity. However, serum diluted at 1:50 inevitably gave unwanted high background interference of non-specific binding.

Besides the prerequisite product specificity, the primary criterion for the optimal dilution of the conjugated detector antibody, HRP-AHG should exhibit a high signal-to-noise ratio, i.e. perfect sensitivity to detect minute amount of specific analyte, and yet generate low background from the $F(ab')_2$ preparation with likely residual Fc contamination. The ideal ratio of positive signal to background at working dilution should be 10:1 or greater[118].

The empirical optimization of coating $F(ab')_2$ quantitation, blocking and diluting agent concentration, serum analyte dilution and conjugated detector antibody titration in the ELISA established the rigid experimental setting for the subsequent investigation of anti-Ids against anti-HLA antibodies.

Chapter 5

Quality Control

5. Quality Control

5.1. Avoidance of Prozone Phenomenon

Comparison of mean OD at 492 nm, generated from quadruple analyses, of a serum sample known to have high anti-F(ab')$_2$ reactivity and a normal control serum, was made at dilutions from 1:50 to 1:400. Data from microplate wells which were coated with 0.2 ug anti-HLA F(ab')$_2$ and detected with 1:30000 Fc-specific HRP-AHG conjugate, showed that there was no evidence of any prozone phenomenon (Figure 5.1). Two-component curves, with a comparatively rapid decline from 1:50 to 1:100 dilution and an apparent flattening of OD from dilutions of 1:200 onwards, were again observed. The pre-determined optimized ELISA setting demonstrated a clear discrimination between strong and weak signals without any unwanted prozone phenomena.

5.2. Inter-assay and Intra-assay Precision

The intra-assay precision was evaluated by performing 18 to 23 replicate analyses of a control serum with high anti-F(ab')$_2$ reactivity in a single batch, on microplate wells coated separately with 100 uL of 9 anti-HLA F(ab')$_2$ preparations of 2 ug/mL (Table 5.1). The inter-assay precision was basically performed in parallel with test samples and tested by examining the same

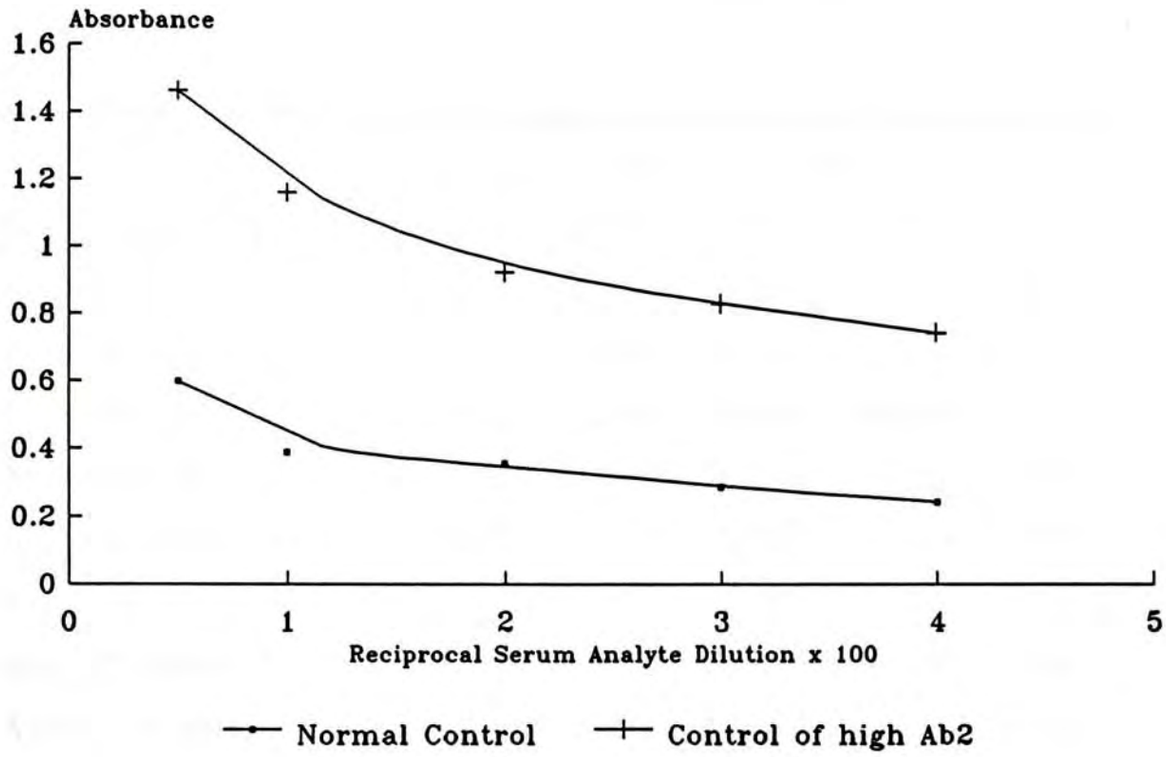


Figure 5.1. Titration and comparison of a serum analyte with high anti-F(ab')$_2$ activity and a normal control serum.

Table 5.1. Intra-assay Precision on control serum sample.

	Intra-assay Precision				
Anti-Idiotypes against	n	OD	SD	SEM	%CV
Anti-HLA-A11	18	1.139	0.051	0.012	4.47
Anti-HLA-A24	19	1.040	0.070	0.016	6.73
Anti-HLA-A2+A28	22	1.307	0.044	0.009	3.36
Anti-HLA-B17	20	1.155	0.052	0.012	4.50
Anti-HLA-Bw22+B7	21	1.320	0.059	0.013	4.46
Anti-HLA-B40+B13	22	1.140	0.069	0.015	6.05
Anti-HLA-DR2	18	1.023	0.037	0.009	3.61
Anti-HLA-DR4	23	1.210	0.033	0.007	2.72
Anti-HLA-DR7	19	1.118	0.062	0.014	5.54

where :

n = number of tests
OD = Mean Absorbance
SD = Standard Deviation
SEM = Standard Error of Mean
%CV = Coefficient of Variation in percentage

control serum of high anti-$F(ab')_2$ reactivity 10 to 11 times, duplicate on each occasion, for the 9 anti-HLA $F(ab')_2$ preparations (Table 5.2).

The reproducibility of the whole project was properly guarded. The %CV values given by the control serum on 9 coating anti-HLA $F(ab')_2$ ligands were well below 10; ranging from 2.72 to 6.73 for intra-assay and 3.70 to 8.00 for inter-assay. There was also no significant difference in the mean OD of the control serum for both intra-assay and inter-assays in the 9 different ligands ($p > 0.05$).

5.3. Discussion

Antigen-antibody complex formation is subject to the prozone phenomena. When antigen in appropriate quantity is allowed to react with a fixed amount of antibody, a point is reached at which the immune complex appears most rapidly and abundantly. Conversely, if an increasingly large amount of antigen is permitted to react with a decreasing quantity of antibody, a point is reached at which no immune complex is visualized. It is important since the non-appearance of immune complex in such experimental setting may be erroneously regarded as negative or weak end-point. Employment of suitable or multiple dilutions can avoid these untoward phenomena.

Table 5.2. Inter-assay precision on control serum sample.

	Inter-assay Precision				
Anti-Idiotypes against	n	OD	SD	SEM	%CV
Anti-HLA-A11	22	1.181	0.067	0.014	5.67
Anti-HLA-A24	22	1.161	0.043	0.009	3.70
Anti-HLA-A2+A28	22	1.255	0.062	0.014	4.94
Anti-HLA-B17	20	1.194	0.073	0.016	6.11
Anti-HLA-Bw22+B7	22	1.307	0.053	0.011	4.05
Anti-HLA-B40+B13	22	1.192	0.065	0.014	5.45
Anti-HLA-DR2	20	1.079	0.047	0.011	4.35
Anti-HLA-DR7	20	1.250	0.100	0.022	8.00

where :

n = number of tests
OD = Mean Absorbance
SD = Standard Deviation
SEM = Standard Error of Mean
%CV = Coefficient of Variation in percentage

Data in Figure 5.1 show that the pre-determined experimental setting, including the dilution of serum analyte at 1:100, was so designed that prozone phenomenon was unlikely to go undetected, and good differentiation between strong and weak reactions could be achieved within sensitive working OD range, without any compromises in sensitivity of the relatively weak end-points presumably generated by normal controls.

Due to the non-availability of suitable commercial and in-house anti-Ids standards, the absolute quantitation of anti-Ids was rendered impossible in this project. Instead, a semi-quantitative analysis, based upon the numeric values of OD generated by the captured Anti-Ids, was adopted to investigate the level of anti-Ids production in the study groups. The significance of the OD values was put into perspective by comparing them with those from normal controls. The occurrence of relatively higher Anti-Ids in transfused renal transplant patients was so determined. Statistical manipulation was subsequently made to explore possible correlation of Anti-Ids level with rejection episodes and graft survival.

Enzyme immunoassay is vulnerable to experimental deviation and inconsistency. The intra-assay and inter-assay precision in this study played a paramount role in ensuring that the OD values were representative of the anti-Ids levels.

Chapter 6

Adjustment of Anti-casein Interference

6. Adjustment of Anti-casein Interference

It is known that anti-casein can be found in the general population, and may be especially to those who are intolerant to dairy products[119]. These anti-casein antibodies have been known to cause interference in detecting humoral antibodies by the ELISA. As part of this project, anti-casein antibodies were investigated, so that their interference in the analyses of anti-Ids could be eliminated.

6.1. Casein Allergy

Casein is the principal protein of milk, the basis of curd and cheese. It belongs to the family of phospho-protein in which the phosphate prosthetic group esterifies to serine residues of its protein back-bone. It is precipitated from milk as a white amorphous substance by dilute acids, however redissolves on the addition of alkalis or an excess of acid. Rennet and calcium change it to an insoluble curd.

More or less, all ingested food proteins are likely to be allergenic to a certain degree, though they normally lead to no seriously adverse effects. Food ingestant-induced nominal minor allergic discomfort may take on a variety of forms; such as nausea, vomiting and diarrhoea. Factors dominating the severity

of allergy are the level of sensitization, type of allergic sensitivity, extent of allergen absorption from gut and antibody isotype as well. Among the broad spectrum of ingested food allergens, casein is one of the culprits[119].

The type of protein in diet may influence directly the intrinsic capacity of B lymphocytes to respond to an immunogenic stimulus[120]. Formation of antibodies against casein may reflect a normal natural humoral response. Recently Harris and his co-workers in Pennsylvania studied the ontogeny of the antibody response to cow milk proteins on 29 infants and found that breast-fed infants demonstrated significantly lower level of these specific antibodies than infants who took milk formula or breast milk with formula supplementation[121].

Though precise data on the intolerance of cow milk and/or milk products are not available in Hong Kong ethnic Chinese, an anecdotal high incidence has been widely speculated. The postulation comes from the apparent dislike of dairy products in diets among Chinese.

The majority of the inhabitants of Hong Kong were mostly economic and political migrants. They were ethnic Han and Hakka Chinese from the southern provinces of mainland China during the second world war in the early 40s followed by the civil war. During those years and years after, the social economical environment in Hong Kong was primitive, and breast-feeding was the only form of nutrition available to infants. Subsequent immunization to

casual dairy products during their adolescence would be obvious.

In more recent years, because of tremendous booming of the local economy and the progressive improvement of the standard of living as well as the impact of "westernization", breast-feeding hardly further sustained its popularity. Formula milk feeding was gradually preferred in the sense that it allows mothers being gainfully employed to subsidize the family incomes. Formula milk feeding also represents a status symbol for young mothers of the modern era. Hence, dairy products are relatively new to the gastro-intestinal system among ethnic Chinese in Hong Kong.

Physiological and genetic factors, together with the fact that Chinese are, in general, intolerant to dairy foodstuffs, are reasons to presume that the prevalence of anti-casein among Hong Kong Chinese is high.

6.2. Prevalence of Anti-casein

A semi-qualitative method was adopted to analyze the presence of anti-casein in each of the 31 serum samples from normal controls and 151 serum samples from the 101 transplant patients studied. Duplicate analyses were performed in parallel with the detection of anti-Ids against 9 Ab_2 (see Method section 2.2.4). The OD of OPD, from a total of 18 replicate analyses on 9

occasions, generated by the putative anti-casein in each serum analyte, were read at 492 nm against the reagent blank.

An arbitrary OD value greater than 0.01, after reagent blank adjustment, which is well beyond 3 SD of mean OD value of the negative samples (0.001 ± 0.006), was established in this project as the cut-off point to indicate the presence of anti-casein antibody.

Table 6.1 shows the prevalence of anti-casein with mean OD reading greater than 0.01. Relatively strong anti-casein was encountered in LD transplant group. The overall incidence of anti-casein detected was 23.48% (31/132). In both CD and LD transplant recipients, the percentage of anti-casein encountered was 24.75% (25/101).

6.3. Discussion

Inference drawn from the data showed that ELISA-detectable anti-casein antibodies were commonly encountered in the Hong Kong Chinese population. In order to make adjustment for the interference caused by the putative anti-casein antibodies in the anti-Ids studies, dilutions of serum analytes were made in 0.5% PBSC. Anti-casein antibodies would be neutralized and inhibited. In addition, background OD generated by anti-casein was determined for each

Table 6.1 Prevalence of anti-casein in normal control group and renal transplant patients.

	Anti-casein detected		
	Control	CD Graft	LD Graft
Number of studies	31	48	53
Number of studies with OD > 0.01	5	10	14
Maximum OD encountered	0.120	0.144	0.693
Mean OD from group with OD > 0.01	0.065	0.073	0.173
Percentage of Anti-casein	19.35	20.83	28.30

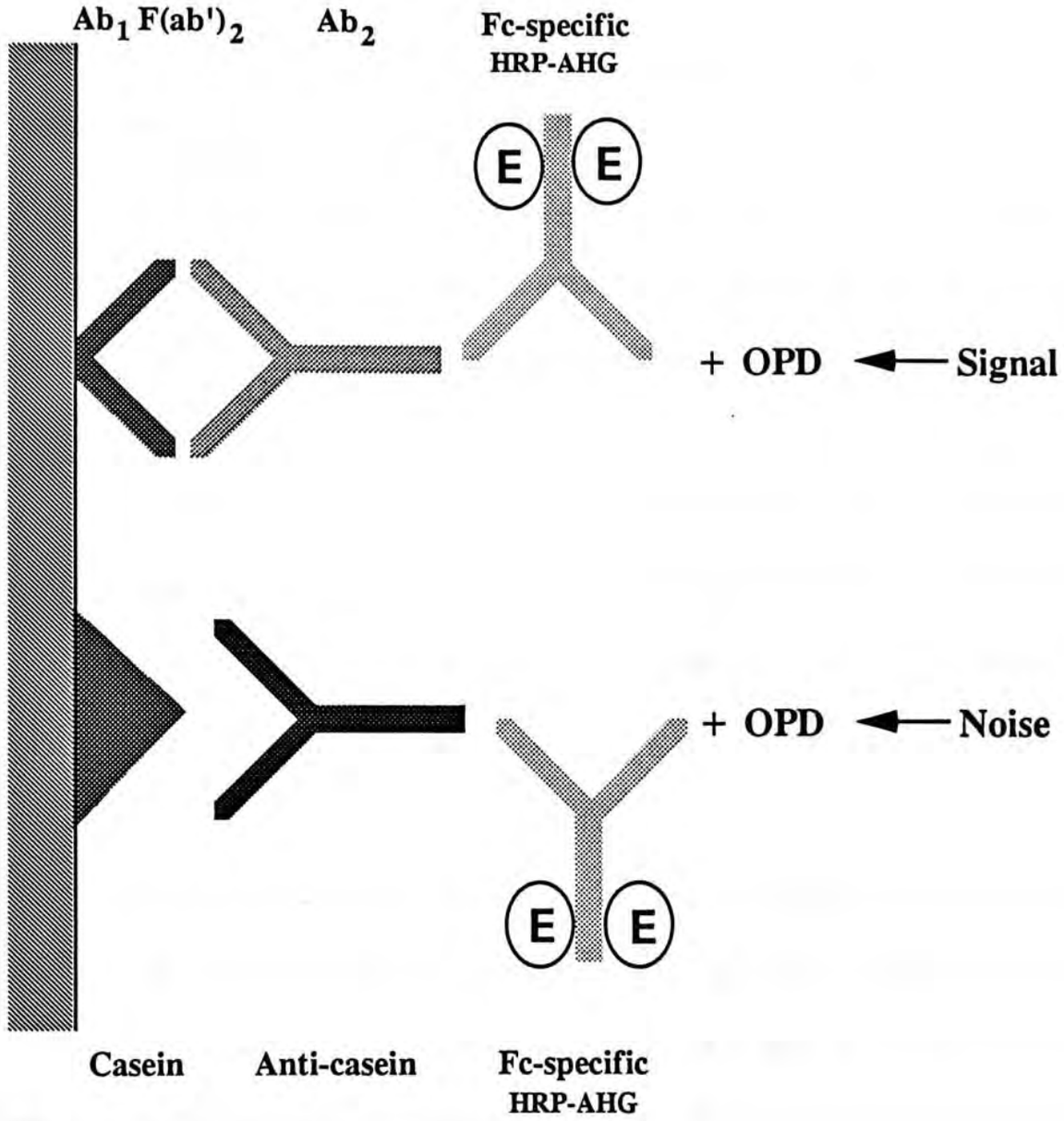


Figure 6.1. The interference caused by putative anti-casein in serum analyte should be eliminated from the signal generated by Ab_2.

serum studied, and was subtracted to yield a genuine OD reading for the anti-Ids present (Figure 6.1).

There have been reports on the association of morbidity with the detection of anti-casein. Recently the correlation of circulating immune complexes containing bovine proteins and graft-versus host disease is noted[122]. Transplant recipients with graft-versus-host disease are found more likely to have higher antibodies against bovine casein and circulating casein immune complexes than patients without this complication.

In untreated coeliac patients, comparatively elevated titre of antibodies to casein is encountered. Moreover, significantly higher quantities of antibody to casein are observed even in treated coeliac patients as compared with the normal controls[123].

Effort was made in this project to investigate whether there was any association of anti-casein immunization to end-stage renal failure requiring transplantation. The χ^2 analysis for 2 x 2 contingency table in Table 6.2 ($\chi^2 = 0.744$; $p > 0.25$) demonstrates that there was insufficient evidence to reject the null hypothesis. Anti-casein immunization and end-stage renal failure requiring transplantation were independent variables and not related.

Table 6.2. Chi-squared test for 2 x 2 contingency table ($\chi^2 = 0.384$; $p > 0.25$) shows that there is no correlation of anti-casein immunization to end-stage renal failure requiring transplantation.

	Anti-casein detected		
Renal Transplantation	**Yes**	**No**	**Total**
Yes	**25**	**76**	**101**
No	**6**	**25**	**31**
Total	**31**	**101**	**132**

Chapter 7

Prevalence

of

Anti-idiotypic Antibodies

7. Prevalence of Anti-idiotypic Antibodies

7.1. Formation Kinetics

A total of 5436 anti-Ids assays were done on 151 serum samples from 101 renal graft recipients against 9 different anti-HLA idiotypes. Temporal study was feasible in 26 (3 not transfused and 23 transfused) patients, whose weekly serum samples were available for periodic anti-Ids assays. The number of days, at which serum samples generated the highest level of anti-Ids as measured by OD at 492 nm, was plotted into a histogram showing the percentage of patients with peak anti-Ids level at the same time interval after transplantation (Figure 7.1). A right-skew pattern of distribution was observed with pinnacle at post-transplant time interval at 51 to 100 days, then followed by a gentle decline and eventually a level off. Thirty eight percent (10/26) of the peak anti-Ids detected were encountered in the early 100 days after transplantation.

7.2. Occurrence in Transplant Patients

Anti-Ids assays were done in quadruple on each of the 76 serum samples of 48 patients transplanted with CD kidney and 75 serum specimen of 53 LD renal graft recipients. For those patients whose temporal study of anti-Ids were permissible, mean value from quadruple analyses of serum samples giving the

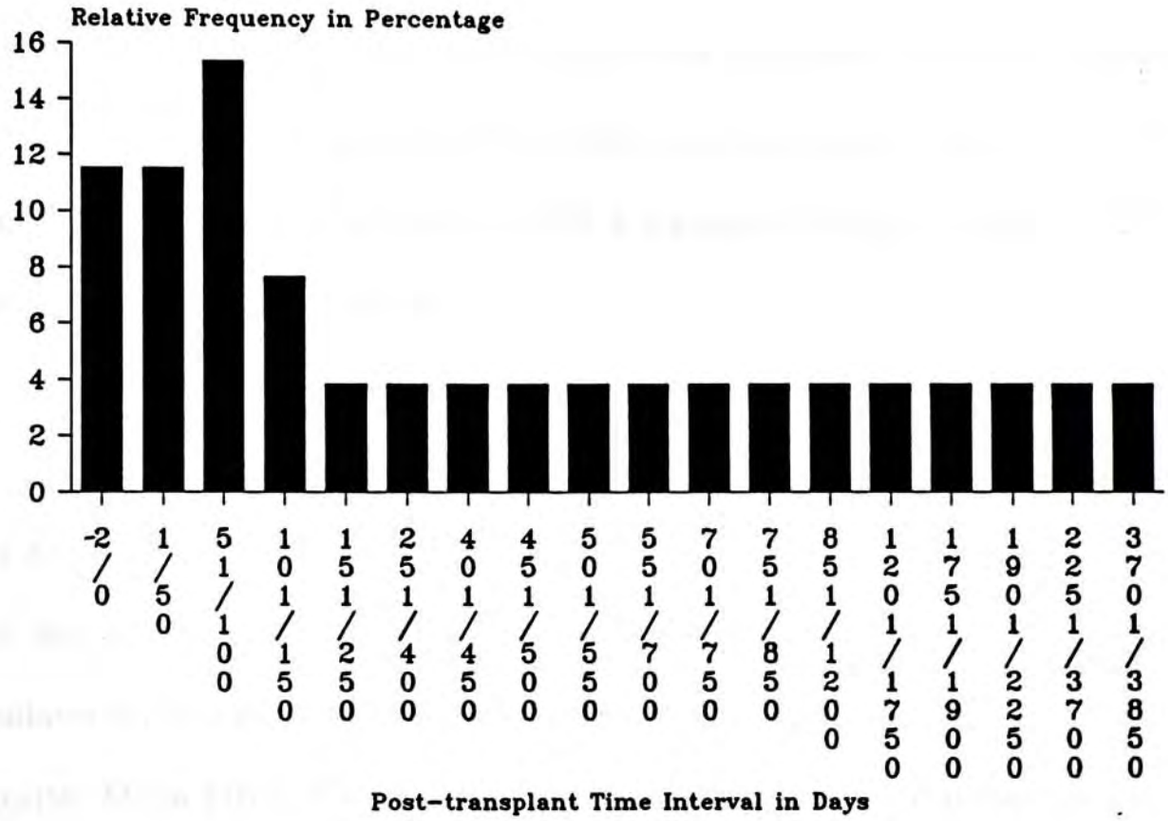


Figure 7.1. The distribution of 26 studies with peak anti-Ids level dectected at various time intervals before and after renal transplant.

highest OD was enrolled into the statistical manipulation for comparison. The levels of anti-Ids against 9 anti-HLA antibodies which were chosen to cover the most frequent antigens of HLA-A, HLA-B and HLA-DR loci in both CD and LD renal grafts, were compared with 31 apparently healthy normal subjects who had never been transfused and received any organ transplant (Table 7.1 - 7.9). Comparisons of anti-Ids prevalence were also made in patients receiving kidney grafts specific to each of the 9 Ab_1 and the patients receiving random renal grafts of known and unknown HLA typing with those of normal controls of random HLA phenotyping.

In the investigation of anti-Ids against idiotopes of anti-HLA antibodies to antigens in HLA-A locus, data in Table 7.1 show that significantly high level of anti-Ids against anti-HLA-A11 was encountered in HLA-A11 negative, cadaveric renal graft recipients either receiving mismatched HLA-A11 positive grafts (Mean OD 0.774 vs 0.322; $p < 0.01$) or grafts of random antigen in HLA-A locus (0.572 vs 0.322; $p < 0.01$). However, in HLA-A11 negative, LD renal graft recipients who were transplanted with HLA-A11 positive kidney, insignificant increase of anti-Ids was noted in comparison with normal control (0.525 vs 0.322; $p < 0.1$). On the other hand, the HLA-A11 negative, LD transplant patients receiving renal grafts of random antigen in HLA-A locus, were found exhibiting notably high level of anti-Ids against idiotopes of anti-HLA-A11 antibody (0.577 vs 0.322; $p < 0.002$).

Table 7.1. Comparison of OD of anti-Ids against anti-HLA-A11 antibody in HLA-A11 negative renal transplant patients with OD from normal controls of random HLA phenotypes.

	HLA-A Typing				
	Control	Cadaver		Living Donor	
	Random	A11	Random	A11	Random
n	31	3	33	5	26
Mean OD	0.322	0.774	0.572	0.525	0.577
Variance	0.055	0.079	0.144	0.036	0.109
Statistics	NA*	3.145	3.191	1.834	3.301
p Value	NA	<0.01	<0.01	<0.1	<0.002

* Not applicable

There was no significant elevation of anti-Ids in both CD and LD kidney recipients in whom recipient-donor combination was mismatched for HLA-A24 and/or other HLA-A locus antigens (Table 7.2).

In recipient-donor combination with HLA-A2 or HLA-A28 or both disparity (Table 7.3), increased quantity of anti-Ids was found in patients transplanted with HLA-A2 or HLA-A28 positive CD grafts ($p < 0.05$) and grafts of random antigen in HLA-A locus ($p < 0.02$). On the contrary, comparable amounts of anti-Ids were observed in LD transplant patients after transplantation of either HLA-A2 or HLA-A28 positive grafts (0.193 vs 0.387; $p > 0.1$) or other HLA-A locus disparate antigens (0.433 vs 0.387; $p < 0.1$).

In the analyses of anti-Ids against idiotopes of anti-HLA antibodies to antigens in HLA-B locus, CD and LD kidney transplant recipients of negative HLA-B17, who had been transplanted with HLA-B17 positive grafts or grafts of other HLA-B typings, had no significant elevation ($p > 0.1$) in anti-Ids against anti-HLA-B17 as compared with normal controls (Table 7.4).

The quantitative comparison of anti-Ids against anti-HLA-Bw22+B7 antibody in HLA-Bw22 negative or HLA-B7 negative or both negative renal transplant patients is shown in Table 7.5. In patients transplanted with HLA-Bw22 positive or HLA-B7 positive CD kidney, significantly high level of anti-Ids was detected (0.589 vs 0.353; $p < 0.05$). Similar to HLA-Bw22 positive and

Table 7.2. Comparison of OD of anti-Ids against anti-HLA-A24 antibody in HLA-A24 negative renal transplant patients with OD from normal controls of random HLA phenotypes.

	HLA-A Typing				
	Control	Cadaver		Living Donor	
	Random	A24	Random	A24	Random
n	31	9	40	3	42
Mean OD	0.317	0.488	0.430	0.189	0.374
Variance	0.042	0.118	0.086	0.020	0.073
Statistics	NA*	1.875	1.909	1.050	1.025
p Value	NA	<0.1	<0.1	>0.1	>0.1

* Not applicable

Table 7.3. Comparison of OD of anti-Ids against anti-HLA-A2+A28 antibody in HLA-A2 negative or HLA-A28 negative or both negative renal transplant patients with OD from normal controls of random HLA phenotypes.

	HLA-A Typing				
	Control	Cadaver		Living Donor	
	Random	A2/A28	Random	A2/A28	Random
n	31	5	48	5	53
Mean OD	0.387	0.657	0.563	0.193	0.433
Variance	0.063	0.124	0.126	0.015	0.121
Statistics	NA*	2.115	2.579	1.681	1.89†
p Value	NA	<0.05	<0.02	>0.1	<0.1

* Not applicable
† *F* distribution

Table 7.4. Comparison of OD of anti-Ids against anti-HLA-B17 antibody in HLA-B17 negative renal transplant patients with OD of normal controls of random HLA phenotypes.

	HLA-B Typing				
	Control	Cadaver		Living Donor	
	Random	B17	Random	B17	Random
n	31	5	43	7	48
Mean OD	0.301	0.362	0.362	0.358	0.313
Variance	0.075	0.112	0.081	0.142	0.085
Test Statistics	NA*	0.449	0.930	0.464	0.185
p Value	NA	>0.1	>0.1	>0.1	>0.1

* Not applicable

Table 7.5. Comparison of OD of anti-Ids against anti-HLA-Bw22+B7 antibody in HLA-Bw22 negative or HLA-B7 negative or both negative renal transplant patients with OD of normal controls of random HLA phenotypes.

	HLA-B Typing				
	Control	Cadaver		Living Donor	
	Random	Bw22 /B7	Random	Bw22 /B7	Random
n	31	5	48	3	53
Mean OD	0.353	0.589	0.605	0.598	0.601
Variance	0.052	0.098	0.143	0.057	0.132
Test Statistic	NA*	2.044	3.693	1.772	3.841
p Value	NA	<0.05	<0.001	<0.1	<0.001

* Not applicable

HLA-B7 positive grafts, grafts of random antigen in the HLA-B locus were also capable to elicit anti-Ids response against public cross-reactive idiotopes resided on anti-HLA-Bw22+B7, in both CD kidney recipients (0.605 vs 0.353; $p < 0.001$) and LD transplant patients (0.601 vs 0.353; $p < 0.001$).

In the study of anti-Ids against anti-HLA-B40+B13, modest increase of anti-Ids was encountered in LD transplant patients as compared with those of the control group (Table 7.6). In addition to HLA-B40 positive and HLA-B13 positive grafts, patients receiving CD grafts of random HLA-B antigens have significantly elevated anti-Ids detected ($p < 0.05$).

In the examination of anti-Ids against the idiotopes of anti-HLA antibodies to antigens in HLA-DR locus, there were no significant elevations of anti-Ids to anti-HLA-DR2, in both CD and LD kidney recipients whom recipient-donor combination was mismatched for HLA-DR2 and/or other HLA-DR locus antigens (Table 7.7). However, notably increase of anti-Ids against the idiotopes of anti-HLA-DR4 developed in both CD graft recipients (0.431 vs 0.307; $p < 0.05$) and LD transplant patients (0.497 vs 0.307; $p < 0.01$) of negative HLA-DR4 receiving grafts of random HLA-DR antigen.

In the appraisal of anti-Ids against anti-HLA-DR7 (Table 7.9), significant raise of anti-Ids was observed in patients receiving HLA-DR7 positive LD grafts (0.776 vs 0.351; $p < 0.01$). Irrespective of the graft HLA-DR typing, both

Table 7.6. Comparison of OD of anti-Ids against anti-HLA-B40+B13 antibody in HLA-B40 negative or HLA-B13 negative or both negative renal transplant patients with OD from normal controls of random HLA phenotypes.

	HLA-B Typing				
	Control	Cadaver		Living Donor	
	Random	B40/ B13	Random	B40/ B13	Random
n	31	3	47	6	50
Mean OD	0.367	0.548	0.509	0.467	0.483
Variance	0.061	0.115	0.112	0.046	0.100
Test Statistics	NA*	1.180	2.153	0.924	1.842
p Value	NA	>0.1	<0.05	>0.1	<0.1

* Not applicable

Table 7.7. Comparison of OD of anti-Ids against anti-HLA-DR2 antibody in HLA-DR2 negative renal transplant patients with OD from normal controls of random HLA phenotypes.

	HLA-DR Typing				
	Control	Cadaver		Living Donor	
	Random	DR2	Random	DR2	Random
n	31	8	43	2	45
Mean OD	0.293	0.468	0.364	0.263	0.330
Variance	0.042	0.088	0.071	0.020	0.069
Test Statistics	NA*	1.960	1.295	0.202	0.688
p Value	NA	<0.1	>0.1	>0.1	>0.1

* Not applicable

Table 7.8. Comparison of OD of anti-Ids against anti-HLA-DR4 antibody in HLA-DR4 negative renal transplant patients with OD from normal controls of random HLA phenotypes.

	HLA-DR Typing			
	Control	Cadaver	Living Donor	
	Random	Random	DR4	Random
n	31	35	2	40
Mean Absorbance	0.307	0.431	0.312	0.497
Variance	0.047	0.075	0.004	0.093
Test Statistics	NA*	2.050	0.032	3.066
p Value	NA	<0.05	>0.1	<0.01

* Not applicable

Table 7.9. Comparison of OD of anti-Ids against anti-HLA-DR7 antibody in HLA-DR7 negative renal transplant patients with OD from normal controls of random HLA phenotypes.

	HLA-DR Typing				
	Control	Cadaver		Living Donor	
	Random	DR7	Random	DR7	Random
n	31	2	47	3	51
Mean OD	0.351	0.629	0.525	0.776	0.485
Variance	0.059	0.245	0.128	0.015	0.103
Statistics	NA*	1.495	2.558	2.964	2.139
p Value	NA	>0.1	<0.02	<0.01	<0.05

* Not applicable

recipients of CD or LD renal grafts experienced significantly high level of anti-Ids to idiotopes of anti-HLA-DR7.

Patients receiving CD renal graft were more predisposed to anti-Ids activation in idiotypic-antiidiotypic interactions than LD renal graft recipients. Table 7.10 shows that the prevalence of significant increase of anti-Ids to anti-HLA antibodies specific to the graft HLA antigens was more frequently encountered in CD kidney recipients than LD renal transplant patients (3/8 vs 1/9). The same also applied to kidney transplantation of random grafts rather than planned HLA-matched grafts (6/9 vs 4/9).

7.3. Transfusion effect

Blood transfusion was regarded here either as the infusion of either random (third party) donations of whole blood, plasma-reduced blood and/or platelet concentrates anticoagulated with CPDA-1 solution supplied by Hong Kong Red Cross Blood Transfusion Centre or related donor-specific (single donor) donations. Other blood products; fresh frozen plasma and cryo-precipitates, were not defined as blood transfusion in this project. Buffy coats were given to anyone in the patient population studied.

Primarily transfusion was evaluated under the all or none basis in this

Table 7.10. Summary of the prevalence of anti-Ids against the 9 anti-HLA antibodies in CD and LD kidney recipients.

	Anti-idiotypic antibodies against								
	Anti-HLA-A			Anti-HLA-B			Anti-HLA-DR		
	11	24	2+28	17	w22+7	40+13	2	4	7
HLA-specific									
LD Graft	-	-	-	-	-	-	-	-	+
CD Graft	+	-	+	-	+	-	-	NA	-
Random HLA									
LD Graft	+	-	-	-	+	-	-	+	+
CD Graft	+	-	+	-	+	+	-	+	+

- Not different from normal controls at 5% significance level
+ Different from normal controls at 5% significance level
NA Not applicable

project. The association of transfusion on anti-Ids response in renal transplant patients was studied and compared under these stipulations.

7.3.1. Comparison between Transfused Transplant Patients and Normal Controls

The effect of blood transfusion on the induction of ant-Ids response in renal transplant recipients was studied according to the various transfusion modalities namely: pre-transplant transfusion, peroperative transfusion and post-transplant transfusion. The quantitation, as defined by the magnitude of OD, of anti-Ids against each of the 9 anti-HLA antibodies in the transplant patients manipulated with different transfusion modalities, was compared with normal subjects who have never been transfused and transplanted (Table 7.11 - 7.19).

Significantly high level of anti-Ids against anti-HLA-A11 was observed in pre-transplant transfused and post-transplant transfused CD kidney recipients. Conversely no significant increase in the level of anti-Ids was encountered in non-transfused CD kidney recipients (Table 7.11), hence transplantation *per se* did not appear to trigger an significantly high anti-Ids response. Comparable anti-Ids level was obtained also in peroperatively transfused CD kidney recipients ($p > 0.10$). In LD transplant recipients a

Table 7.11. Comparison of OD of anti-Ids against anti-HLA-A11 antibody in transfused and non-transfused HLA-A11 negative renal transplant patients with OD from normal controls of random HLA phenotypes.

	Control	Cadaveric Transplant					Living Donor Related Transplant			
Transfusion	No	Yes[1]	Pre[2]	Per[3]	Post[4]	No	Yes	Pre	Non-Pre[5]	No
No. of Cases	31	25	14	4	7	8	20	19	1	6
Mean OD	0.322	0.601	0.654	0.284	0.675	0.484	0.574	0.535	1.315	0.586
Variance	0.055	0.149	0.129	0.085	0.156	0.120	0.117	0.093	NA	0.083
Test Statistics	NA[6]	3.336	3.707	0.298	3.147	1.575	3.125	2.778	NA	2.437
p Value	NA	<0.002	<0.001	>0.10	<0.01	>0.10	<0.01	<0.01	NA	<0.05

1 Anyone of the transfusion modalities
2 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
3 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
4 Post-transplant transfusion only.
5 All modalities except pre-transplant transfusion.
6 Not applicable.

higher anti-Ids level developed in both transfused and non-transfused patients when compared with normal controls. There was insufficient evidence to indicate, one way or the other, whether transfusion was responsible for the increased anti-Ids formation.

Table 7.12 shows the transfusion effect on the enhancement of anti-Ids to anti-HLA-A24 antibody. Significantly higher anti-Ids quantity was detected in pre- and post-transplant transfused CD kidney recipients ($p < 0.05$), but not in peroperatively transfused patients ($p > 0.10$). The level of anti-Ids detected in non-transfused CD kidney recipients was slightly higher than that of normal controls (0.380 vs 0.317), and did not reach a statistically significant level ($p > 0.10$). Also, there was no significant difference of anti-Ids in transfused and non-transfused LD transplant patients ($p > 0.10$).

Similar findings occurred in anti-Ids against anti-HLA-A2+A28 (Table 7.13), with the exception that non-pre-transplant transfused LD recipients acquired significantly elevated anti-Ids (0.820 vs 0.424; $p < 0.02$).

For anti-Ids against anti-HLA-B17 antibodies, comparable OD values with $p > 0.10$ were observed in both transfused and non-transfused CD and LD transplant recipients (Table 7.14), inferring that there was insufficient evidence to reject the null hypothesis that blood transfusion and increased anti-Ids against anti-HLA-B17 were independent and not related.

Table 7.12. Comparison of OD of anti-Ids against anti-HLA-A24 antibody in transfused and non-transfused HLA-A24 negative renal transplant patients with OD from normal controls of random HLA phenotypes.

	Control	Cadaveric Transplant					Living Donor Related Transplant			
Transfusion	No	Yes[1]	Pre[2]	Per[3]	Post[4]	No	Yes	Pre	Non-Pre[5]	No
No. of Cases	31	30	18	5	7	10	36	34	2	6
Mean OD	0.317	0.447	0.462	0.227	0.563	0.380	0.388	0.382	0.493	0.291
Variance	0.042	0.091	0.067	0.035	0.144	0.066	0.078	0.072	0.158	0.033
Test Statistics	NA[6]	1.975	2.166	0.920	2.420	0.795	1.170	1.090	1.134	0.289
p Value	NA	<0.10	<0.05	>0.10	<0.05	>0.10	>0.10	>0.10	>0.10	>0.10

1 Anyone of the transfusion modalities
2 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
3 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
4 Post-transplant transfusion only.
5 All modalities except pre-transplant transfusion.
6 Not applicable.

Table 7.13. Comparison of OD of anti-Ids against anti-HLA-A2+A28 antibody in transfused and non-transfused, HLA-A2 negative, or HLA-A28 negative, or both negative renal transplant patients with OD from normal controls of random HLA phenotypes.

	Control	Cadaveric Transplant					Living Donor Related Transplant			
Transfusion	No	Yes[1]	Pre[2]	Per[3]	Post[4]	No	Yes	Pre	Non-Pre[5]	No
No. of Cases	31	38	23	7	8	10	45	42	3	8
Mean OD	0.387	0.587	0.625	0.376	0.665	0.470	0.424	0.396	0.820	0.481
Variance	0.063	0.132	0.125	0.069	0.156	0.093	0.121	0.099	0.263	0.118
Test Statistics	NA[6]	2.599	2.895	0.104	2.469	0.863	1.92†	0.131	2.606	0.875
p Value	NA	<0.02	<0.01	>0.10	<0.02	>0.10	<0.10	>0.10	<0.02	>0.10

1 Anyone of the transfusion modalities
2 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
3 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
4 Post-transplant transfusion only.
5 All modalities except pre-transplant transfusion.
6 Not applicable.
† *F* distribution.

Table 7.14. Comparison of OD of anti-Ids against anti-HLA-B17 antibody in transfused and non-transfused HLA-B17 negative renal transplant patients with OD from normal controls of random HLA phenotypes.

	Control	Cadaveric Transplant					Living Donor Related Transplant			
Transfusion	No	Yes[1]	Pre[2]	Per[3]	Post[4]	No	Yes	Pre	Non-Pre[5]	No
No. of Cases	31	33	20	6	7	10	40	38	2	8
Mean OD	0.301	0.366	0.410	0.182	0.401	0.348	0.308	0.293	0.598	0.336
Variance	0.075	0.083	0.091	0.092	0.086	0.075	0.090	0.075	0.276	0.064
Test Statistics	NA[6]	0.924	1.334	0.959	0.862	0.472	0.101	0.121	1.426	0.327
p Value	NA	>0.10	>0.10	>0.10	>0.10	>0.10	>0.10	>0.10	>0.10	>0.10

1 Anyone of the transfusion modalities
2 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
3 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
4 Post-transplant transfusion only.
5 All modalities except pre-transplant transfusion.
6 Not applicable.

Table 7.15 shows significant increase of anti-Ids against anti-HLA-Bw22+B7 in pre-transplant transfused ($p < 0.001$) and post-transplant transfused ($p < 0.01$) CD kidney recipients. No significant increase of anti-Ids level was seen in peroperatively transfused CD kidney recipients ($p > 0.10$). Anti-Ids level in non-transfused CD kidney recipients was higher, but the elevation did not reach a statistically significant level (0.508 vs 0.353; $p < 0.10$). Furthermore, in LD transplant recipients statistically significant increase in anti-Ids levels were developed in both the transfused and non-transfused patients.

There were no significant increase, in terms of OD values, of anti-Ids against anti-HLA-B40+B13 in both transfused and non-transfused LD transplant patients as compared with normal controls, though a small cluster of non-pre-transplant transfused patients showed statistically significant elevation of anti-Ids (Table 7.16). This would probably be due to sampling bias, which could also be observed in anti-Ids against anti-HLA-DR2 (Table 7.17) and anti-HLA-DR7 (Table 7.19).

In the study of anti-Ids to anti-HLA-DR4 (Table 7.18), significantly high OD values were observed in both transfused CD and LD kidney recipients. Non-transfused CD and LD transplant patients showed comparable level of anti-Ids to anti-HLA-DR4 to that of normal control (0.323 vs 0.307; $p > 0.1$, 0.462 vs 0.502; $p > 0.10$).

Table 7.15. Comparison of OD of anti-Ids against anti-HLA-Bw22+B7 antibody in transfused and non-transfused HLA-Bw22 negative, or HLA-B7 negative or both negative renal transplant patients with OD from normal controls of random HLA phenotypes.

	Control	Cadaveric Transplant					Living Donor Related Transplant			
Transfusion	No	Yes[1]	Pre[2]	Per[3]	Post[4]	No	Yes	Pre	Non-Pre[5]	No
No. of Cases	31	38	23	7	8	10	45	42	3	8
Mean OD	0.353	0.630	0.675	0.394	0.708	0.508	0.590	0.566	0.932	0.665
Variance	0.052	0.152	0.137	0.093	0.184	0.097	0.125	0.106	0.266	0.164
Test Statistics	NA[6]	3.495	3.945	0.404	3.227	1.706	3.287	3.119	3.745	2.908
p Value	NA	<0.001	<0.001	>0.10	<0.01	<0.10	<0.002	<0.01	<0.001	<0.01

1 Anyone of the transfusion modalities
2 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
3 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
4 Post-transplant transfusion only.
5 All modalities except pre-transplant transfusion.
6 Not applicable.

Table 7.16. Comparison of OD of anti-Ids against anti-HLA-B40+B13 antibody in transfused and non-transfused HLA-B40 negative, or HLA-B13 negative, or both negative renal transplant patients with OD from normal controls of random HLA phenotypes.

	Control	Cadaveric Transplant					Living Donor Related Transplant			
Transfusion	No	Yes[1]	Pre[2]	Per[3]	Post[4]	No	Yes	Pre	Non-Pre[5]	No
No. of Cases	31	37	22	7	8	10	43	40	3	7
Mean OD	0.367	0.535	0.574	0.334	0.602	0.414	0.473	0.447	0.819	0.541
Variance	0.061	0.119	0.113	0.065	0.140	0.076	0.105	0.083	0.262	0.064
Test Statistics	NA[6]	2.267	2.587	0.318	2.150	0.509	1.528	1.234	2.756	1.677
p Value	NA	<0.05	<0.02	>0.10	<0.05	>0.10	>0.10	>0.10	<0.01	>0.10

1 Anyone of the transfusion modalities
2 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
3 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
4 Post-transplant transfusion only.
5 All modalities except pre-transplant transfusion.
6 Not applicable.

Table 7.17. Comparison of OD of anti-Ids against anti-HLA-DR2 antibody in transfused and non-transfused HLA-DR2 negative renal transplant patients with OD from normal controls of random HLA phenotypes.

	Control	Cadaveric Transplant					Living Donor Related Transplant			
Transfusion	No	Yes[1]	Pre[2]	Per[3]	Post[4]	No	Yes	Pre	Non-Pre[5]	No
No. of Cases	31	34	21	6	7	9	38	36	2	7
Mean OD	0.293	0.395	0.410	0.225	0.494	0.246	0.325	0.305	0.686	0.359
Variance	0.042	0.078	0.069	0.029	0.109	0.029	0.070	0.049	0.311	0.063
Test Statistics	NA[6]	1.665	1.802	0.761	2.083	0.626	0.552	0.229	2.393	0.739
p Value	NA	>0.10	<0.10	>0.10	<0.05	>0.10	>0.10	>0.10	<0.05	>0.10

1 Anyone of the transfusion modalities
2 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
3 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
4 Post-transplant transfusion only.
5 All modalities except pre-transplant transfusion.
6 Not applicable.

Table 7.18. Comparison of OD of anti-Ids against anti-HLA-DR4 antibody in transfused and non-transfused HLA-DR4 negative renal transplant patients with OD from normal controls of random HLA phenotypes.

	Control	Cadaveric Transplant					Living Donor Related Transplant			
Transfusion	No	Yes[1]	Pre[2]	Per[3]	Post[4]	No	Yes	Pre	Non-Pre[5]	No
No. of Cases	31	25	14	5	6	10	35	32	3	5
Mean OD	0.307	0.474	0.529	0.245	0.537	0.323	0.502	0.470	0.845	0.462
Variance	0.047	0.075	0.041	0.068	0.104	0.059	0.100	0.076	0.224	0.043
Test Statistics	NA[6]	2.548	3.243	0.578	2.196	0.197	2.884	2.603	3.693	1.491
p Value	NA	<0.02	<0.01	>0.10	<0.05	>0.10	<0.01	<0.02	<0.001	>0.10

1 Anyone of the transfusion modalities
2 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
3 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
4 Post-transplant transfusion only.
5 All modalities except pre-transplant transfusion.
6 Not applicable.

Table 7.19. Comparison of OD of anti-Ids against anti-HLA-DR7 antibody in transfused and non-transfused HLA-DR7 negative renal transplant patients with OD from normal controls of random HLA phenotypes.

	Control	Cadaveric Transplant					Living Donor Related Transplant			
Transfusion	No	Yes[1]	Pre[2]	Per[3]	Post[4]	No	Yes	Pre	Non-Pre[5]	No
No. of Cases	31	37	22	7	8	10	44	41	3	7
Mean OD	0.351	0.550	0.607	0.314	0.598	0.432	0.479	0.454	0.816	0.520
Variance	0.059	0.135	0.136	0.052	0.146	0.088	0.111	0.099	0.146	0.049
Test Statistics	NA[6]	2.579	3.049	0.368	2.267	0.869	1.823	1.513	3.030	1.687
p Value	NA	<0.02	<0.01	>0.10	<0.05	>0.10	<0.10	>0.10	<0.01	>0.10

1 Anyone of the transfusion modalities
2 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
3 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
4 Post-transplant transfusion only.
5 All modalities except pre-transplant transfusion.
6 Not applicable.

Table 7.20 shows the summary of occurrence of significant increase in level of anti-Ids against 9 anti-HLA antibodies encountered in CD and LD transplant patients. Transfusion-induced enhancement of anti-Ids was more frequently observed in CD transplant patients than LD transplant patients (6/9 vs 3/9). Pre-transplant (7/9) and post-transplant (8/9) transfusion were both proficient in triggering the production of anti-Ids; however, peroperative transfusion was totally ineffective (0/9). Non-transfused CD kidney recipients had statistically insignificant high anti-Ids levels (8/9) when compared with the non-transfused, non-transplanted normal controls. On the other hand, significantly high anti-Ids levels in non-transfused LD transplant patients were encountered in 2 out of the 9 anti-HLA antibodies studied. Whether this increase of anti-Ids level to the 2 anti-HLA antibodies was due to sampling bias or to a unique phenomenon which outweighed the transfusion effect in LD transplant patients, could not be ascertained.

7.3.2. Comparison between Transfused Transplant Patients and Non-transfused Transplant Patients

Table 7.11 to Table 7.19 have clearly shown that significantly elevated levels of anti-Ids were observed in transfused renal transplant patients but comparable and statistically insignificant levels of anti-Ids were detected in non-transfused transplant patients when compared with normal controls. It would

Table 7.20. Summary of the comparison of the levels of anti-Ids against 9 anti-HLA antibodies in renal transplant recipients in different transfusion modalities with normal controls.

	Anti-idiotypic antibodies against																	
	Anti-HLA-A						Anti-HLA-B						Anti-HLA-DR					
	11		24		2+28		17		w22+7		40+13		2		4		7	
Transfusion Modality	C	L	C	L	C	L	C	L	C	L	C	L	C	L	C	L	C	L
Not Transfused	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-
Transfused	+	+	-	-	+	-	-	-	+	+	+	-	-	-	+	+	+	-
Pre-transplant transfused	+	+	+	-	+	-	-	-	+	+	+	-	-	-	+	+	+	-
Peroperatively transfused	-	*	-	*	-	*	-	*	-	*	-	*	-	*	-	*	-	*
Post-transplant transfused	+	*	+	*	+	*	-	*	+	*	+	*	+	*	+	*	+	*

C Cadaveric kidney recipients
L Living donor related transplant recipients
- Not different from normal controls at 5% significance level
+ Different from normal controls at 5% significance level
* Not applicable.

be interesting to further expand the observation of transfusion effect on anti-Ids induction in transfused and non-transfused transplant patients.

Table 7.21 to Table 7.29 show the comparison of anti-Ids level in transfused and non-transfused CD and LD kidney recipients, and the impact of the various transfusion modalities on anti-Ids formation. Anti-Ids against 9 anti-HLA antibodies in transfused CD transplant patients were all higher than those of their non-transfused counterparts, though the increase did not reach the 5% significance level. On the contrary, statistically insignificant lower anti-Ids levels in LD transplant recipients were found in 7 out of the 9 anti-HLA antibodies studied.

7.3.3. Association with Graft Survival

Transfusion-induced anti-Ids and their association with renal allograft survival could only be evaluated in CD transplant recipients. The similar study in LD transplant patients was not applicable due to small patient clusters after statistical stratifications, despite data of graft failure and transfusion status were available.

Table 7.30 shows the comparison of anti-Ids to the 9 anti-HLA antibodies in transfused CD transplant patients with respective to graft success and failure.

Table 7.21. Comparison of OD of anti-Ids against anti-HLA-A11 antibody in HLA-A11 negative, transfused transplant patients with OD of non-transfused transplant patients.

	Cadaveric Transplant						Living Donor Related Transplant				
Transfusion	No	Yes[1]	Y&S[2]	Pre[3]	Per[4]	Post[5]	No	Yes	Y&S	Pre	Non-Pre[6]
Number of Cases	8	25	21	14	4	7	6	20	20	19	1
Mean Absorbance	0.484	0.601	0.552	0.654	0.284	0.675	0.586	0.574	0.574	0.535	1.315
Variance	0.120	0.149	0.160	0.129	0.085	0.156	0.083	0.117	0.117	0.093	NA
Test Statistics	NA[7]	0.763	0.423	1.081	0.987	0.998	NA	0.078	0.078	0.361	NA
p Value	NA	>0.10	>0.10	>0.10	>0.10	>0.10	NA	>0.10	>0.10	>0.10	NA

1 Anyone of the transfusion modalities
2 Survived graft with transfusion
3 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
4 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
5 Post-transplant transfusion only.
6 All modalities except pre-transplant transfusion.
7 Not applicable.

Table 7.22. Comparison of OD of anti-Ids against anti-HLA-A24 antibody in HLA-A24 negative, transfused transplant patients with OD of non-transfused transplant patients.

	Cadaveric Transplant						Living Donor Related Transplant				
Transfusion	No	Yes[1]	Y&S[2]	Pre[3]	Per[4]	Post[5]	No	Yes	Y&S	Pre	Non-Pre[6]
Number of Cases	10	30	25	18	5	7	6	36	35	34	2
Mean Absorbance	0.380	0.447	0.445	0.462	0.227	0.563	0.291	0.388	0.389	0.382	0.494
Variance	0.066	0.091	0.105	0.067	0.035	0.144	0.033	0.078	0.080	0.072	0.158
Test Statistics	NA[7]	0.629	0.566	0.805	1.176	1.191	NA	0.818	0.815	0.795	1.072
p Value	NA	>0.10	>0.10	>0.10	>0.10	>0.10	NA	>0.10	>0.10	>0.10	>0.10

1 Anyone of the transfusion modalities
2 Survived graft with transfusion
3 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
4 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
5 Post-transplant transfusion only.
6 All modalities except pre-transplant transfusion.
7 Not applicable.

Table 7.23. Comparison of OD of anti-Ids against anti-HLA-A2+A28 antibody in HLA-A2 negative, or HLA-A28 negative or both negative, transfused transplant patients with OD of non-transfused transplant patients.

	Cadaveric Transplant						Living Donor Related Transplant				
Transfusion	No	Yes[1]	Y&S[2]	Pre[3]	Per[4]	Post[5]	No	Yes	Y&S	Pre	Non-Pre[6]
Number of Cases	10	38	31	23	7	8	8	45	44	42	3
Mean Absorbance	0.470	0.587	0.572	0.625	0.376	0.665	0.481	0.424	0.420	0.396	0.820
Variance	0.093	0.132	0.146	0.125	0.069	0.156	0.118	0.121	0.123	0.099	0.263
Test Statistics	NA[7]	0.933	0.767	1.203	0.660	1.184	NA	0.428	0.454	0.691	1.292
p Value	NA	>0.10	>0.10	>0.10	>0.10	>0.10	NA	>0.10	>0.10	>0.10	>0.10

1 Anyone of the transfusion modalities
2 Survived graft with transfusion
3 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
4 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
5 Post-transplant transfusion only.
6 All modalities except pre-transplant transfusion.
7 Not applicable.

Table 7.24. Comparison of OD of anti-Ids against anti-HLA-B17 antibody in HLA-B17 negative, transfused transplant patients with OD of non-transfused transplant patients.

	Cadaveric Transplant						Living Donor Related Transplant				
Transfusion	No	Yes[1]	Y&S[2]	Pre[3]	Per[4]	Post[5]	No	Yes	Y&S	Pre	Non-Pre[6]
Number of Cases	10	33	27	20	6	7	8	40	39	38	2
Mean Absorbance	0.348	0.366	0.371	0.410	0.182	0.401	0.336	0.308	0.306	0.293	0.598
Variance	0.075	0.083	0.095	0.091	0.092	0.086	0.064	0.090	0.092	0.075	0.276
Test Statistics	NA[7]	0.175	0.207	0.546	1.129	0.382	NA	0.246	0.261	0.408	1.102
p Value	NA	>0.10	>0.10	>0.10	>0.10	>0.10	NA	>0.10	>0.10	>0.10	>0.10

1 Anyone of the transfusion modalities
2 Survived graft with transfusion
3 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
4 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
5 Post-transplant transfusion only.
6 All modalities except pre-transplant transfusion.
7 Not applicable.

Table 7.25. Comparison of OD of anti-Ids against anti-HLA-Bw22+B7 antibody in HLA-Bw22 negative, or HLA-B7 negative, or both negative, transfused transplant patients with OD of non-transfused transplant patients.

	Cadaveric Transplant						Living Donor Related Transplant				
Transfusion	No	Yes[1]	Y&S[2]	Pre[3]	Per[4]	Post[5]	No	Yes	Y&S	Pre	Non-Pre[6]
Number of Cases	10	38	31	23	7	8	8	45	44	42	3
Mean Absorbance	0.508	0.630	0.619	0.675	0.394	0.708	0.665	0.590	0.589	0.566	0.932
Variance	0.097	0.152	0.166	0.137	0.093	0.184	0.164	0.125	0.128	0.106	0.266
Test Statistics	NA[7]	0.913	0.788	1.245	0.749	1.147	NA	0.541	0.542	0.759	0.913
p Value	NA	>0.10	>0.10	>0.10	>0.10	>0.10	NA	>0.10	>0.10	>0.10	>0.10

1 Anyone of the transfusion modalities
2 Survived graft with transfusion
3 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
4 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
5 Post-transplant transfusion only.
6 All modalities except pre-transplant transfusion.
7 Not applicable.

Table 7.26. Comparison of OD of anti-Ids against anti-HLA-B40+B13 antibody in HLA-B40 negative, or HLA-B13 negative, or both negative, transfused transplant patients with OD of non-transfused transplant patients.

	Cadaveric Transplant						Living Donor Related Transplant				
Transfusion	No	Yes[1]	Y&S[2]	Pre[3]	Per[4]	Post[5]	No	Yes	Y&S	Pre	Non-Pre[6]
Number of Cases	10	37	30	22	7	8	7	43	42	40	3
Mean Absorbance	0.414	0.535	0.516	0.574	0.334	0.602	0.541	0.473	0.477	0.447	0.819
Variance	0.076	0.119	0.129	0.113	0.065	0.140	0.064	0.105	0.107	0.083	0.262
Test Statistics	NA[7]	1.022	0.819	1.314	0.607	1.229	NA	0.528	0.492	0.809	1.196
p Value	NA	>0.10	>0.10	>0.10	>0.10	>0.10	NA	>0.10	>0.10	>0.10	>0.10

1 Anyone of the transfusion modalities
2 Survived graft with transfusion
3 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
4 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
5 Post-transplant transfusion only.
6 All modalities except pre-transplant transfusion.
7 Not applicable.

Table 7.27. Comparison of OD of anti-Ids against anti-HLA-DR2 antibody in HLA-DR21 negative, transfused transplant patients with OD of non-transfused transplant patients.

	Cadaveric Transplant						Living Donor Related Transplant				
Transfusion	No	Yes[1]	Y&S[2]	Pre[3]	Per[4]	Post[5]	No	Yes	Y&S	Pre	Non-Pre[6]
Number of Cases	9	34	28	21	6	7	7	38	37	36	2
Mean Absorbance	0.246	0.395	0.405	0.410	0.225	0.494	0.359	0.325	0.326	0.305	0.686
Variance	0.029	0.078	0.090	0.069	0.029	0.109	0.063	0.070	0.072	0.049	0.311
Test Statistics	NA[7]	1.519	1.505	1.716	0.234	1.956	NA	0.315	0.301	0.579	1.300
p Value	NA	>0.10	>0.10	>0.10	>0.10	<0.10	NA	>0.10	>0.10	>0.10	>0.10

1 Anyone of the transfusion modalities
2 Survived graft with transfusion
3 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
4 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
5 Post-transplant transfusion only.
6 All modalities except pre-transplant transfusion.
7 Not applicable.

Table 7.28. Comparison of OD of anti-Ids against anti-HLA-DR4 antibody in HLA-DR4 negative, transfused transplant patients with OD of non-transfused transplant patients.

	Cadaveric Transplant						Living Donor Related Transplant				
Transfusion	No	Yes[1]	Y&S[2]	Pre[3]	Per[4]	Post[5]	No	Yes	Y&S	Pre	Non-Pre[6]
Number of Cases	10	25	20	14	5	6	5	35	34	32	3
Mean Absorbance	0.323	0.474	0.479	0.529	0.245	0.537	0.462	0.502	0.510	0.470	0.845
Variance	0.059	0.075	0.084	0.041	0.068	0.104	0.043	0.100	0.101	0.076	0.224
Test Statistics	NA[7]	1.518	1.461	2.262	0.573	1.512	NA	0.273	0.326	0.062	1.631
p Value	NA	>0.10	>0.10	<0.05	>0.10	<0.10	NA	>0.10	>0.10	>0.10	>0.10

1 Anyone of the transfusion modalities
2 Survived graft with transfusion
3 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
4 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
5 Post-transplant transfusion only.
6 All modalities except pre-transplant transfusion.
7 Not applicable.

Table 7.29. Comparison of OD of anti-Ids against anti-HLA-DR7 antibody in HLA-DR7 negative, transfused transplant patients with OD of non-transfused transplant patients.

	Cadaveric Transplant						Living Donor Related Transplant				
Transfusion	No	Yes[1]	Y&S[2]	Pre[3]	Per[4]	Post[5]	No	Yes	Y&S	Pre	Non-Pre[6]
Number of Cases	10	37	30	22	7	8	7	44	43	41	3
Mean Absorbance	0.432	0.550	0.534	0.607	0.314	0.598	0.520	0.479	0.481	0.454	0.816
Variance	0.088	0.135	0.150	0.136	0.052	0.146	0.049	0.111	0.113	0.099	0.146
Test Statistics	NA[7]	0.934	0.759	1.316	0.883	1.039	NA	0.313	0.295	0.531	1.585
p Value	NA	>0.10	>0.10	>0.10	>0.10	<0.10	NA	>0.10	>0.10	>0.10	>0.10

1 Anyone of the transfusion modalities
2 Survived graft with transfusion
3 Four transfusion modalities, namely: pre-transplant transfusion, pre-transplant transfusion + peroperative transfusion, pre-transplant transfusion + post-transplant transfusion, or the combination of all 3 modalities.
4 Peroperative transfusion and peroperative transfusion + post-transplant transfusion.
5 Post-transplant transfusion only.
6 All modalities except pre-transplant transfusion.
7 Not applicable.

Table 7.30. Comparison of OD of anti-Ids in transfused CD kidney recipients with respect to graft success and failure. Patients were negative of the HLA to which the 9 anti-HLA antibodies directed.

Anti-idiotypes to	Cadaveric Transplantation with Transfusion						Test Statistics	*p* Value
	Graft Survival			Graft Failure				
	n	OD	Var	n	OD	Var		
Anti-HLA-A11	21	0.552	0.160	4	0.855	0.014	-1.038	0.299
Anti-HLA-A24	25	0.445	0.105	5	0.452	0.023	-0.036	0.718
Anti-HLA-A2+A28	31	0.572	0.146	7	0.657	0.061	-0.734	0.463
Anti-HLA-B17	27	0.371	0.095	6	0.345	0.025	-0.420	0.674
Anti-HLA-Bw22+B7	31	0.619	0.166	7	0.681	0.086	-0.470	0.638
Anti-HLA-B40+B13	30	0.516	0.129	7	0.614	0.067	-0.970	0.332
Anti-HLA-DR2	28	0.405	0.090	6	0.345	0.016	-0.045	0.964
Anti-HLA-DR4	20	0.479	0.084	5	0.455	0.040	-0.068	0.946
Anti-HLA-DR7	30	0.534	0.150	7	0.619	0.065	-0.776	0.438

where n = number of grafts, OD = mean absorbance and Var = variance

Patients were negative for the HLA to which each of the 9 anti-HLA antibodies reacted. The anti-Ids levels were in general comparable. There was insufficient evidence to reject the null hypothesis at 5% significance level: anti-Ids in transfused patients with successful grafts should be higher than those in transfused patients with graft failure.

Comparisons of anti-Ids levels were also made in transfused and non-transfused CD kidney recipients with graft failures (Table 7.31). Anti-Ids to anti-HLA-A11 was the only anti-Ids which showed statistically significant difference (0.855 vs 0.440; $p < 0.05$).

Anti-Ids levels in transfused transplant patients with successful grafts and non-transfused kidney recipients with graft failure were also compared (Table 7.32) and found to be comparable ($p > 0.10$). There was again insufficient evidence to reject the null hypothesis at 5% significance level; indicating that anti-Ids in transfused patients with successful grafts were not higher than those in non-transfused patients with graft failure.

7.4. Discussion

In Jerne's immune network concepts[4], anti-Ids are, in fact, immuno-suppressive in nature, and act as surveillance agents to monitor the homeostasis

Table 7.31. Comparison of OD of anti-Ids in failed CD kidney recipients with respect to transfusion. Patients were negative of the HLA to which the 9 anti-HLA antibodies directed.

Anti-idiotypes to	Failed Cadaveric Renal Transplantation						Test Statistics	*p* Value
	Transfused			Not Transfused				
	n	OD	Var	n	OD	Var		
Anti-HLA-A11	4	0.855	0.014	4	0.440	0.102	-1.061	0.289
Anti-HLA-A24	5	0.452	0.023	3	0.433	0.097	-0.149	0.882
Anti-HLA-A2+A28	7	0.657	0.061	3	0.400	0.083	-1.026	0.305
Anti-HLA-B17	6	0.345	0.025	3	0.222	0.019	-1.033	0.302
Anti-HLA-Bw22+B7	7	0.681	0.086	3	0.413	0.055	-1.026	0.305
Anti-HLA-B40+B13	7	0.614	0.067	3	0.353	0.061	-1.254	0.210
Anti-HLA-DR2	6	0.345	0.016	3	0.276	0.046	-0.516	0.606
Anti-HLA-DR4	5	0.455	0.040	3	0.262	0.035	-1.044	0.297
Anti-HLA-DR7	7	0.619	0.065	3	0.453	0.133	-0.570	0.569

where n = number of patients, OD = mean absorbance and Var = variance

Table 7.32. Comparison of OD of anti-Ids in transfused CD transplant patients with graft survival and non-transfused CD transplant patients with graft failure. Patients were negative of the HLA to which the 9 anti-HLA antibodies directed.

Anti-idiotypes to	Transfused with Graft Survival			Non-transfused with Graft Failure			Test Statistics	*p* Value
	n	OD	Var	n	OD	Var		
Anti-HLA-A11	21	0.552	0.160	4	0.440	0.102	-0.742	0.458
Anti-HLA-A24	25	0.445	0.105	3	0.433	0.097	-0.186	0.853
Anti-HLA-A2+A28	31	0.572	0.146	3	0.400	0.083	-0.880	0.379
Anti-HLA-B17	27	0.371	0.095	3	0.222	0.019	-0.795	0.427
Anti-HLA-Bw22+B7	31	0.619	0.166	3	0.413	0.055	-0.759	0.448
Anti-HLA-B40+B13	30	0.516	0.129	3	0.353	0.061	-0.814	0.416
Anti-HLA-DR2	28	0.405	0.090	3	0.276	0.046	-0.601	0.548
Anti-HLA-DR4	20	0.479	0.084	3	0.262	0.035	-1.369	0.171
Anti-HLA-DR7	30	0.534	0.150	3	0.453	0.133	-0.438	0.661

where n = number of patients, OD = mean absorbance and Var = variance

of the immune response. The introduction of external antigens disrupts and upsets the state of equilibrium of idiotypic-antiidiotypic interactions. The emergence of the corresponding antibodies specific to the challenging antigens, in turn, activates the proliferation of anti-Ids until the system again returns to its state of stability.

Most of the complementarity-determining amino acid residues of an antibody, which function in binding are the surfaces not directly involved in binding[124]. They are exposed and become available as antigenic determinants. They could very well share determinants with the surfaces of a receptor for the same ligand. Anti-Ids directed against these antibody determinants can cross-react with receptors specific for the same ligand. Data in this study demonstrated the presence of anti-Ids against the public, cross-reactive idiotopes of the 9 anti-HLA antibodies in both the allogeneic renal transplant recipients and the apparently healthy normal subjects, hence they supported Jerne's general immune network theory which utilizes idiotypic determinants to recognize and regulate the immune response.

The maximal level of anti-idiotypic antibodies against anti-HLA antibodies occurred over a range of pre-transplant and post-transplant time intervals. However, according to the limited data in this project, most patients reached their peak anti-Ids level in the first 100 days after transplantation (Figure 7.1). This is also the post-transplant period when the renal allograft is

most susceptible to acute rejection[125]. It is possible that the increase in formation of anti-Ids during such period when renal allograft is so vulnerable to acute rejection, is not merely incidental, but rather it is the way of the immune system to respond to a novel antigenic challenge. This surge in anti-Ids production may play a role in tolerating or rejecting the graft, depending on which way the balance is tipped.

It is not surprising to observe circulating cross-reactive anti-Ids in normal subjects who have no history of transfusion or organ transplantation, considering the abundant naturally occurring substances which can mimic biologically active ligands.

It has been reported that $Ab_{2\beta}$ which direct to the private (unique) idiotopes are particularly low in quantity[126]. Though effort has not been made to differentiate and classify the anti-Ids encountered in this project, data in Table 7.10 show that the incidence of detecting significantly high anti-Ids levels in patients having renal allografts of random HLA was comparatively more frequent as compared with those having HLA specific graft to which the 9 anti-HLA antibodies reacted (6/9 vs 3/8 in CD transplants and 4/9 vs 1/9 in LD transplants). This paradoxical finding can be explained by the likelihood of being sensitized to the public idiotopes in patients having received renal allograft of random HLA. The elicited anti-Ids which directed against the cross-reactive idiotypes of the anti-HLA antibodies studied, would probably be predominated

with $Ab_{2\alpha}$ in nature, and lesser amount of $Ab_{2\beta}$ specific to the paratopes of the 9 anti-HLA antibodies existed. It is possible that the minute quantity of $Ab_{2\beta}$ generated was beyond the sensitivity of the ELISA used in this project. Chemiluminescent enzyme immunoassay[127] which is 100 times more sensitive than ELISA, can be employed as an alternative to detect this scantiness of $Ab_{2\beta}$.

LD transplant patients were found less likely to produce significantly high anti-Ids when compared to CD transplant patients (4/9 in LD transplant vs 6/9 in CD transplant; Table 7.10). This may be attributed to the inferior chance of alloimmunization of anti-HLA antibodies to disparate HLA by the intended HLA matching of donor-recipient pair. The majority of LD transplant are either HLA-identical or single-haplotype identical, and occasional HLA-disparate, in the sense that antigens in HLA-A, B and DR loci can be matched as far as possible and negative MLR is obtained[128]. It is understandable that cross-reactive anti-Ids to public idiotopes of the 9 anti-HLA antibodies did exist in LD transplant recipients, however the planned HLA-matching has restrained the relative predisposition of cross-reactive idiotopes immunization to a great extent.

Interestingly, it has been shown that the beneficial effect of transfusion will disappear if HLA-DR matching is practised in recipient-donor pairs, and the beneficial transfusion effect is most profound when HLA-DR matching is not used[32,40]. Transfusion has been shown to contribute no improvement in graft

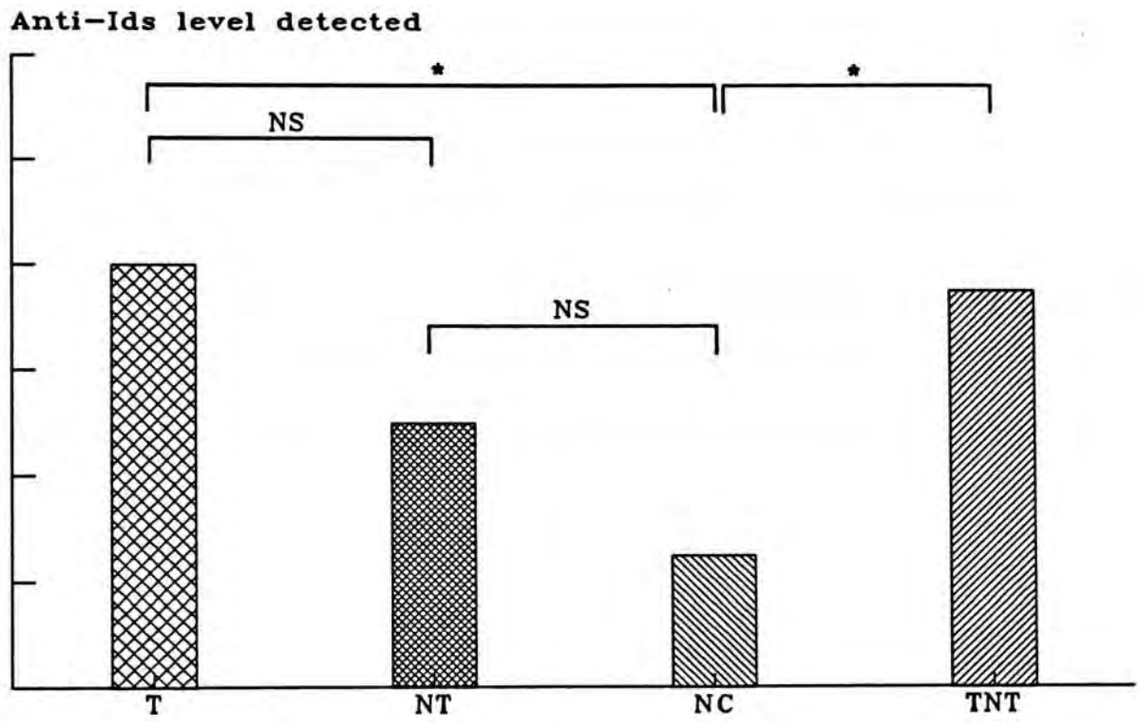
survival in a series of CD kidney transplant patients who had all been HLA-DR matched[129]. The inference is simply that HLA-DR matching overshadows the observation of transfusion effect. For transfusion is regarded as religious convictions as in Jehovah's witnesses[130], and in people simply resent homologous transfusion for fears of blood borne transmissible diseases, attention to HLA-matching between donor and recipient is an alternative to transfusion to achieve the same beneficial goal in enhancing graft survival if transfusion is precluded in renal transplantation.

Goulmy and co-workers[131] reported non-reactivity of post-transplant donor-specific cell-mediated lympholysis in renal allograft recipients with peroperative transfusions. The development of anti-Ids to those donor-specific antibodies in allo-sensitized recipients could contribute to the loss of exhibiting specific anti-HLA antibodies[132]. Furthermore, in a study of peroperative transfusions on the outcome of CD renal transplantation, acceptable graft survival rates were obtained[133].

Data in this study showed that peroperative transfusion was comparatively inferior with its counterparts (pre-transplant transfusion and post-transplant transfusion) in provoking anti-Ids; none of the 18 comparisons in CD renal recipients (Table 7.11 -7.19 and Table 7.21 - 7.29) demonstrated significantly high anti-Ids against the 9 anti-HLA antibodies. The lack of anamnestic response could be the appropriate explanation to this phenomenon.

The introduction of Ab_1 in pre-transplant transfusion acts as primary stimulation in the immune response, and yields bunches of Ab_2 memory clones and small quantity of Ab_2. The subsequent implantation of the renal graft triggers the Ab_2 memory clones and results a significant Ab_2 proliferation. The same explanation can also apply to the increase of anti-Ids in post-transplant transfusion with the allograft as primary stimulation and transfusion as the second.

Apparently the conclusion drawn from data in Table 7.11 to Table 7.19 is incompatible with the inference drawn from data in Table 7.21 to Table 7.29. It appears that the elevation of anti-Ids levels was statistically significant only between transfused transplant patients and healthy subjects, notable but not statistically significant between a) transfused transplant patients and non-transfused transplant patients, and b) non-transfused transplant patients and healthy subjects (Figure 7.2). The simple explanation for this discrepancy is that the increase in anti-Ids level, as measured by ELISA under this experimental setting, is in small increments being not considered as statistically significant. The elevation of anti-Ids level in the transfused transplant patients represents the cumulation of multiple small increments which becomes statistically significant. Hence, response to antigenic stimulation from anti-HLA Ab_1 is a stepwise increment in anti-Ids production. Some increments occur after transfusion; some more after transplant. The prerequisite of the anamnestic response through the subsequent immunological challenge would also



T = Transfused Transplant Patients
NT = Non-Transfused Transplant Patients
NC = Non-Transfused & Not Transplanted Normal Controls
TNT = Transplant Patients irrespective of Transfusion Status
* = Significant Increase
NS = Non-significant Increase

Figure 7.2. Schematic presentation of the transfusion-induced stepwise elevation of anti-Ids in renal transplant patients.

account for the significant increase of anti-Ids observed in transfused transplant patients.

Ab_1-potentiating antibodies (Ab_3) against anti-Ids (Ab_2) might account for the graft failure in transfused transplant patients by potentiating the detrimental action of Ab_1 against Ab_1-specific antigen in the renal graft. In Table 7.31, data suggest that the insignificantly high mean OD values generated by the serum analytes of transfused CD transplant recipients with graft failure, in the anti-Ids assay using anti-HLA Ab_1 as ligand might not be necessarily attributed to the markedly increase of anti-Ids against anti-HLA Ab_1. It is possible that the profound proliferation of the Ab_1-potentiating antibodies, Ab_3, in the form of Ab_2-Ab_3 circulating immune complex, can account for this observation.

Chapter 8

Correlation of Transfusion with the Outcome of Transplant

8. Correlation of Transfusion with the Outcome of Transplant

8.1. Rejection Episode

Graft rejection referred in this project included acute and chronic rejections. Clinical presentations involved persistent oligoanuria and azotemia[125].

The histologic findings in post-transplant renal biopsy diagnosed as acute cellular rejection involved tubulointerstitial nephritis with oedema and cortical infiltration by lymphocytes, plasma cells, histiocytes and immunoblasts, and invasion of the tubule basement membrane as well as tubule necrosis. Acute vascular rejection was characterized in renal biopsy by foci of fibrinoid necrosis or intimal fibrin deposition in the walls of arterioles and small arteries.

In chronic rejection, progressive azotemia, proteinuria and hypertension were the clinical hallmarks. Histologically glomeruli were small with wrinkled or thickened basement membranes or increased mesangial matrix. Some glomeruli were completely hyalinized or sclerosed. Vascular changes included intimal proliferation with luminal obliteration and medial necrosis with degenerative changes of the internal elastic lamina. Tubule atrophy with luminal obliteration and interstitial fibrosis were also seen.

Patients with complete record of rejection episodes and transfusion history were assorted into 2 groups according to their transfusion history; transfused and non-transfused. Table 8.1 shows that rejection happened more frequent in non-transfused than transfused cadaveric kidney recipients ($p < 0.05$). There was no significant difference of rejection episodes encountered in LD kidney recipients ($p > 0.05$). The overall rejection episodes were significantly higher in non-transfused transplant patients ($p < 0.01$).

8.2. Graft Survival

Graft failure was considered to have occurred when patients returned to maintenance dialysis therapy, graft nephrectomy, or death with or without a functioning graft.

Of 101 transplant patients studied, the graft failure rates were 5.66% (3/52) and 20.83% (10/48) for LD renal allograft recipients and CD kidney recipients respectively. The overall failure rate was 12.87% (13/101). CD kidney recipients were 3.68 times more likely to experience graft failure as compared to their LD transplant counterparts (10/48 in CD transplant vs 3/53 in LD transplant). Chi-squared analysis of the data in Table 8.2 shows that graft survival was related to transfusion ($p < 0.05$).

Table 8.1. Comparison of the rejection episodes in transfused and non-transfused renal graft recipients.

	Rejection Episodes							
	Transfused			Not Transfused				
Renal Transplantation	n	Mean	Var	n	Mean	Var	*F*- Test Statistics	*p* Value
Cadaver	27	1.888	1.062	3	2.6	5.55	5.226	<0.05
Living Donor	29	1.724	0.889	2	1	0	0	>0.05
Overall	56	1.803	0.979	5	2	2	4.086	<0.01

where n = number of transplants, Mean = average number of rejection episodes per patient and Var = variance.

Table 8.2. Chi-squared test for 2 x 2 contingency table ($\chi^2 = 4.340$; $p < 0.05$) shows that graft survival is related to transfusion.

	Transfusion		
Graft Status	**Yes**	**No**	**Total**
Survival	**75**	**13**	**88**
Failure	**8**	**5**	**13**
Total	**83**	**18**	**101**

8.3. Discussion

Interestingly, the average number of rejection episodes per patient in this study was comparatively higher than those reported elsewhere[134]. The increased graft survival has been found to result from decreased graft rejection, and not apparently related to other differences among patients receiving various types of blood[36]. The finding of reduced acute rejection after 1 or more random transfusions suggests that a non-specific effect is induced by crossing a major histocompatibility antigen barrier[135]. Susceptibility to early renal allograft rejection episodes in renal transplantation of Jehovah's witnesses is another evidence of the beneficial effect of transfusion reducing rejection episodes[130]. On the contrary, Melzer and co-workers showed that transfusions did not alter the number of rejection episodes, but did reduce the severity of rejections[136].

An increased peak MLR to donor and/or third-party has been shown to link with early ($\leq$ 3 days) rejection crises, and a significant proportion of transplant patients with donor-specific transfusion plus azathioprine-immunosuppression had a decreased MLR response[56]. Elevation of serum C-reactive protein levels during graft rejection is also reported[137].

Scornik and co-workers[138] demonstrated that anti-donor antibodies, predominantly IgG, were detected in 40% post-transplant patients close to the time of rejection and 9% of non-rejecting patients, but these antibodies were

exclusively IgM. Post-transplant cytotoxic antibody reactivity against panel cells developed or increased in some patients, yet it did not correlate with rejection. Previous sensitization and post-transplant transfusions favour the development of post-transplant panel reactivity but not of anti-donor antibodies. Anti-donor antibodies are postulated as being the culprit involved in a significant proportion of rejection episodes, however the damage induced does not necessarily culminate in the loss of the graft.

Not only does the frequency of rejection episodes influence renal graft survival, but also the severity of rejection plays much role. Relatively scarce data exist in the literature concerning the difference in the frequency of rejection episodes between transfused and non-transfused renal transplant patients. In this study there has been convincing data to suggest that rejection episodes are less frequent in the transfused transplant patients (Table 8.1). This information can be valuable to health care professionals in renal transplantation, since it verifies the fact that transfusion can be used as an alternative to the much more expensive anti-rejection drugs when it comes to reducing rejection episodes. The beneficial effect of transfusion in reducing rejection severity[136] would also be another incentive to alleviate rejection complications, hence medical resources could be better utilized.

However, the inference must be considered in the light of the concomitant effect of the immunosuppressive drug on the frequency of rejection

episodes in transfused transplant patients. Azathioprine and prednisolone were conventionally used for prophylactic immunosuppression in the early days (Table 8.3) Starting October of 1987, cyclosporin was then routinely prescribed, in conjunction with prednisolone and/or azathioprine for mismatched CD or LD transplants. In a study, it was shown that the number of rejection episodes per patient after transplantation was significantly higher in patients transfused with 5 units of blood, and were managed with azathioprine and prednisone than those transfused with only 0 to 2 units of blood but given cyclosporin and prednisone, though no statistically significant difference in graft survival existed between them[139].

The total number of renal transplants done in the 3 major public renal dialysis and transplant centres, namely: Princess Margaret Hospital, Queen Mary Hospital and Prince of Wales Hospital, between years 1984 and 1991, was 357 with 152 CD transplants and 205 LD transplants[140,141]. The 101 patients enrolled in this study were either in-patients, out-patients or patients who had had renal transplants done overseas (PRC, USA and UK), and later returned to the medical renal unit of Princess Margaret Hospital for follow-up. Table 8.4 shows the general information of patients recruited in this study. The sample size of this study was 28.29% (101/357) of the total transplants done locally from the inception of the renal transplant programme up to the end of 1991.

It is striking to observe that the graft failure rate in CD transplant was

Table 8.3. Statistics of kidney transplants performed in Hong Kong and the number of patients on haemodialysis in years 1984 - 1991.

Year	Number of Patients on Haemodialysis	Number of Patients Transplanted	Source of Graft	
			Cadaver	Living Donor
1984	394	31	4	27
1985	465	35	13	22
1986	525	40	12	28
1987	674	44	18	26
1988	784	52	17	35
1989	1002	48	24	24
1990	1100	55	32	23
1991	1305	52	32	20

Table 8.4. General information of patients recruited in this study.

	Renal Transplant Patients receiving	
	Cadaver Donor Graft	Living Donor graft
Number of studies	48	53
Ratio of Male to Female	2.43 : 1	2.31 : 1
Age Range (Year)	23 to 54	15 to 53
Graft Survival Range (Year)	¾ to 22	½ to 19

nearly 4-fold higher that of LD transplant. Part of the reason for this observation could be quite a number of patients who had received CD transplant in mainland China, and these recipients might not have received the same standard of care as the local transplant patients. The first year mortality rate of these patients was also 4 times that of transplants done locally[141]. Moreover, there was marked increase in post-transplant morbidity related to chronic hepatitis B and hepatitis C infections[142].

This study confirmed the beneficial effect of transfusion in renal transplantation and supported the hypothesis of transfusion-induced immuno-modulation of anti-Ids in renal transplantation. Transfused renal transplant recipients had significantly lesser rejection episodes and better graft survival than their non-transfused counterparts. The results were also in line with other similar reports[143,136].

Bucin[144] reserved conditionally that enhanced graft survival was only compiled with HLA-A incompatibility and with HLA-B compatibility among transfused patients. In his analysis of the effects of pre-transplant transfusion in 116 patients whose 2-year survival after transplantation were significantly lower in transfused than non-transfused patients, he reported that transfusion might even have detrimental effect on patient survival. Likewise, Kerman and co-workers[145] illustrated that the beneficial transfusion effect could also be achieved in 100 non-transfused CD kidney recipients managed by cyclosporin

and prednisone immunosuppressive therapy. However, they also demonstrated that the matchings of HLA-A, HLA-B and HLA-DR antigens played no role, and immunosuppressive therapy alone was effective in primary CD renal transplantation without the need for pre-transplant transfusion conditioning.

Chapter 9

General Conclusions

9. General Conclusions

Despite general acceptance of the benefits of pre-transplant transfusion and the development of rational conditioning protocols for transfusion, the precise mechanism of the beneficial effect remains vague and obscure. The majority of hypotheses cannot adequately explain all the observed phenomena, and many of them probably only work in certain experimental settings.

The study of transfusion effect, based upon the quantitation of anti-Ids to the 9 anti-HLA antibodies in this project, demonstrated the beneficial effect of blood transfusion in renal transplantation. In addition, the reduced average rejection episodes per patient and the enhancement of graft survival in transfused transplant recipients associated with an increase of anti-Ids levels indicated that idiotypes-anti-idiotypes interactions of the network theory of immune system may be operative in this instance.

Renal dysfunction gives rise to a variety of metabolic and haematologic disturbances, including anaemia, leucocyte dysfunction, and coagulopathy[146]. The anaemia of renal failure has been attributed to a relative deficiency of erythropoietin. Blood transfusion has long been the conventional clinical manifestation to alleviate haematological deficiency in uraemic patients after haemodialysis. Recently, with the advent of recombinant human erythropoietin[147], normalization of haemoglobin can be easily achieved with the

administration of this novel stimulating factor, and transfusion may become obsolete to display its beneficial effect in renal transplantation.

Deliberate blood transfusion which has been employed in patients with renal disease for virtually 2 decades as a pre-conditioning regime for renal transplantation is now under intense criticism and debate. Though the improvement of renal graft survival by the immunosuppression of blood transfusion has been appreciated, there has been growing concern for transfusion transmitted diseases[148], the adverse effects of transfusion in potentiating sepsis[149], disseminating metastasis and enhancing recurrence of malignancy[150]. The availability of effective immunosuppressive agents[151] and the supply of recombinant human erythropoietin[147] as an alternative to transfusion, together with the above concerns related to transfusion lead to the necessity of re-appraisal of transfusion in the pre-transplant conditioning.

Donor specific transfusion and cyclosporin are two available immuno-suppressive strategies to improve graft tolerance in high MLR, single-haplotype matched LD transplant, though each has its own drawbacks. The conventional strategy of donor specific transfusion may sensitize the recipient to donor antigens, precluding transplantation from that donor. Beneficial effect of elective transfusion on allograft survival must be weighed against risks of sensitization. The concomitant administration of cyclosporin in transfusion has been shown to achieve decreased allo-sensitization with no abrogation of anti-Ids

formation[143], however, cyclosporin may escalate graft failure due to nephrotoxicity[152,153].

The beneficial effect of transfusion has lost much its prestige in renal graft enhancement since the start of the cyclosporin era, and its effect on graft survival is currently under continuous scrutiny. Conflicting reports, ranging from demonstrating a beneficial outcome[154] to excellent result[155] without transfusion, have been published. Enhancement of renal graft survival is a multifactorial sequel and the immunosuppressive effect of cyclosporin cannot be singly considered[156]. Better patient care may have also attributed to the improvement. In addition, the nephrotoxicity of cyclosporin can hardly be regarded as a benefit.

This study demonstrated the potential benefit of blood transfusion in renal transplant. There is a political adage which goes "never believe a rumour until it is officially denied". Transfusion conditioning regime of prospective recipients for renal transplantation has been the subject rife with rumours. The denial by some reports of the beneficial effect of transfusion on renal transplantation just might bring it back into the lime-light again.

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